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PROPAGATION OF RABIES VIRUS IN CULTURES OF HAMSTER KIDNEY CELLS[1]

PAUL FENJE

Abstract

A strain of rabies fixed virus has been successfully cultivated in tissue cultures of hamster kidney cells. This confirms an earlier report by Kissling. In the experiments here recorded a special culture tube incorporating a dialyzing membrane made it possible to maintain the cells in continuous culture for many weeks. By using this technique it was possible to obtain culture fluids of high infectivity.

Introduction

In a recent publication Kissling (3) has reported the successful cultivation of rabies virus in tissue cultures of hamster kidney, thus breaking the long series of failures by other workers to propagate the virus in cultures of non-neural cells. The importance of this achievement needs to be stressed. Rabies virus develops very slowly, even in the nerve tissue of its usual hosts; and except for a few strains of fixed virus maintained in experimental animals, it is present only in low titer. The study of the virus is further complicated by its instability outside the host cell and the technical difficulty of separating the infective virus particle from cell constituents. Once the virus has been firmly established in tissue culture, further progress along two lines of investigation can be expected: an increase in our knowledge of the general biological properties of the virus, and a much needed improvement in the anti-rabies vaccines used for human and animal prophylaxis. It was with these two objectives, particularly the second, that an attempt was made to confirm Kissling's work. After many initial failures this was successfully accomplished. The present report is concerned mainly with technical details and with the data on which this claim is based. As this work was nearing completion Kaplan, Forsek, and Koprowski (2) published a short report announcing that they had successfully cultivated a fixed strain of rabies virus in hamster kidney tissue cultures. This report together with our own experimental findings provides ample confirmation of Kissling's original discovery.

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Methods

A strain of rabies fixed virus designated SAD was used for most of the experiments here recorded. This strain was originally isolated from the brain of a rabid dog at the Communicable Disease Centre, Montgomery Laboratory, Alabama, and had been propagated exclusively in mouse brain. The infectivity of the tissue cultures was determined by intracerebral inoculation of the Connaught Laboratories strain of inbred Swiss white mice aged 3 to 4 weeks. The hamsters were young, healthy specimens supplied by a local breeder of laboratory animals. Proof of identity of the virus harvested from the tissue cultures was obtained by cross serum–virus neutralization tests using rabbit antisera to strains SAD and CVS, the latter being one of the standard strains recognized as suitable for this purpose.

Preparation of Tissue Cultures

Suspensions of hamster kidney cells were prepared in the usual manner by tryptic digestion of minced tissue. The nutrient medium was composed of Hanks' balanced salt solution (4) with lactalbumin hydrolyzate 0.5% and serum 15.0%. Human and horse sera were found to give much better results than sera taken from other species of animals. The cells usually formed complete monolayers after incubation at 37° C for 4 to 5 days. A synthetic nutrient medium was used in some of the earlier trials but the simpler medium gave better results. For the first series of experiments the cultures were prepared in tubes or flat bottles which were held stationary or rotated in a drum. The usual infecting dose was the equivalent of 1000 mouse LD_{50} units of virus as determined by intracerebral inoculation of mice.

In the later experiments cultivation of hamster kidney cells in simple tubes or bottles was abandoned in favor of dialyzer tubes. This change was necessary because, in our hands, the ordinary methods failed to provide conditions favorable for proliferation of the virus although they allowed it to survive appreciably longer than in control tubes containing no cells. These dialyzer tubes were similar to those used by Vieuchange (5) for the cultivation of rabies virus. The culture tube is a Pyrex test tube, size 160 mm × 28 mm, provided with a tightly fitting silicone stopper through which passes a piece of glass tubing, size 150 mm × 15 mm. A length of dialysis tubing is slipped over the portion of the glass tube, which protrudes into the culture tube and is tied in place with nylon thread. The lower end of the dialysis tubing, which extends the whole length of the culture tube, is closed by tying in a knot. The upper end of the glass tube is closed with another silicone stopper. The culture tube and the dialyzing assembly are wrapped in paper and sterilized separately. A volume of 10 ml of culture medium is introduced into the tube together with trypsinized kidney cell suspension equivalent to about 250,000 cells. The tube is then incubated at 37° C in a position slightly inclined to the horizontal. When a cell monolayer has formed the medium is changed, the infecting dose of virus is added, and the dialysis assembly is fitted into the culture tube. The dialysis tube is then filled with 25 ml of culture medium and closed with a silicone stopper. The whole apparatus is now ready to be returned to the incubator.

At intervals of 4 to 6 days the fluid in the dialysis tube is removed and replaced by fresh medium: the cell monolayer is not disturbed. In this apparatus it is possible to maintain the cells in a healthy condition for periods up to 10 weeks. The advantages of this method of cultivation for a slowly developing virus such as rabies will be at once apparent. In the method described by Vieu-change the cells were introduced into the dialysis sack and it was the medium outside the membrane which was changed at intervals. By reversing the procedure, the cells are able to adhere to the wall of the glass culture tube: this allows them to be examined under the microscope at low magnification and it also helps to maintain the cells in a favorable environment by providing an air space above the surface of the culture medium.

Results

To adapt an essentially neurotropic virus, such as a fixed strain of rabies, to the very different environment of a kidney cell constitutes a major modification of biological characters. An abrupt and permanent change of environment seemed less likely to prove successful than a more gradual change. With this as a guiding principle, and again following the technique of Kissling (3), a method of alternate cultivation in the two kinds of cell was adopted. Hamster kidney cells were infected with rabies virus in the form of a mouse brain suspension. After a suitable period of incubation the cells were harvested and a clarified suspension was prepared from the culture fluid. This was titrated for virus content by intracerebral inoculation of mice. These animals in turn yielded the virus for infection of a second set of hamster kidney tissue cultures, and so on, through as many alternations as were necessary to adapt the virus to hamster kidney cells.

In the earlier experiments in which a simple culture tube technique was used even this somewhat elaborate method of alternating the host cells failed to yield convincing evidence of in vitro proliferation of the virus. In the control tubes containing nutrient medium without cells the virus disappeared within 3 to 4 days. In the presence of hamster kidney cells the virus survived as long as 9 days but a definite increase of titer could not be demonstrated. In contrast the dialysis tube technique gave unequivocal evidence of proliferation. The experiment described below is a typical example.

Experiment No. 59

The seed virus was a suspension of mouse brain representing the 30th passage of strain SAD in an alternate mouse brain – hamster kidney sequence. The infecting dose, 1000 mouse LD_{50} units, was added to the nutrient medium in contact with the cell monolayer and the culture was incubated at 37° C, with replacement of the fluid in the dialysis tube every 4 days. Samples of the culture fluid in contact with the cells were removed at intervals and titrated immediately by intracerebral inoculation of white mice. A summary of the results is given in Table I.

TABLE I

Experiment No. 59
Growth of rabies virus strain SAD in hamster kidney tissue cultures (dialysis tube method)

Serial No. of sample	Time of removal of sample	Infectivity of culture fluid (mouse brain LD_{50} doses/ml)
1	At start	$10^{4.47}$
2	3 hours	$10^{3.17}$
3	1 day	$10^{2.47}$
4	2 "	$10^{3.47}$
5	3 "	$10^{4.65}$
6	4 "	$10^{5.5}$
7	13 "	$10^{6.21}$
8	20 "	$10^{6.21}$
9	23 "	$10^{7.0}$
10	29 "	$10^{7.0}$
11	36 "	$10^{6.0}$
12	46 "	$10^{5.47}$
13	53 "	$10^{5.23}$
14	62 "	$10^{2.70}$
Control		
1*	At start	$10^{4.47}$
2	3 hours	$10^{4.47}$
3	1 day	$10^{1.95}$
4	2 "	Not infective
5	3 "	"
6	4 "	"

*The control tubes were set up in the same manner as those of the test except that they contained no cell monolayer

At a later stage of these investigations the SAD strain became better adapted to growth in hamster kidney cells and serial transfer in these cells was at length accomplished. The dialysis tube technique was continued. The results of one serial cultivation experiment are presented in Table II.

TABLE II

Rabies virus strain SAD maintained by serial transfers in cultures of hamster kidney cells grown in dialysis tubes

Number of transfers in hamster kidney cells	Period of cultivation of cells after infection (days)	Infectivity of culture fluids (LD_{50} doses/ml)
2nd	12	$10^{5.0}$
2nd	19	$10^{7.0}$
3rd	8	$10^{4.7}$
3rd	15	$10^{4.7}$
4th	8	$10^{5.7}$
5th	6	10^{4}
5th	13	$10^{4.3}$
5th	30	$10^{3.7}$
6th	7	$10^{4.7}$
6th	11	10^{5}
6th	18	10^{5}
7th	8	10^{5}
7th	15	10^{6}

In this experiment the infective agent recovered from the 5th passage in hamster kidney cells was proved to be rabies virus by carrying out a serum–virus neutralization test in mice.

The pathogenicity of the virus was not appreciably altered as the result of adapting it to hamster kidney cells. Mice inoculated intracerebrally with the culture virus developed encephalitis and died 6 or 7 days after injection. In the case of rabbits, however, the incubation period following intracerebral injection was increased to 12 to 13 days. The subsequent course of the infection was typical of that produced by a fixed virus strain of rabies.

The brain tissue of mice and rabbits infected with the adapted strain was examined by the usual methods of histopathology. There was the usual picture of a viral encephalitis. Negri body inclusions were not found, but many of the neurons contained finely granular acidophil material diffusely situated in the cytoplasm. The appearance of the hamster kidney cells in tissue culture was studied by the flying cover glass technique. It was surprising to find very few pathological changes, even in those cells that had been exposed to the virus for periods as long as 3 weeks. This failure to detect cytopathological changes may have been due to the relatively small number of cells actually infected by the virus.

Comment

In this laboratory successful cultivation of a strain of rabies virus has depended upon the use of a modified Vieuchange dialysis tube. It must be emphasized that this is not a difficult technique: it has become a routine procedure and we shall use it until the virus has become so well adapted to hamster kidney cells that it is no longer necessary. Perhaps the SAD strain is particularly exacting with respect to cultural environment, because Kissling (3) using a simpler technique was successful with several strains both of fixed and street virus.

Progress in the study of rabies virus in tissue culture is hindered by the absence of any readily recognizable cytopathogenic changes. Even the electron microscope has failed so far to provide information of value. Animal inoculation experiments have shown that the highest infectivity of the cultures is reached after about 2 weeks of incubation. The lability of the virus even in the presence of a protective colloid precludes any accumulation of infective agent. The use of the fluorescent antibody technique, already established as a valuable diagnostic method (1), may lead to a better understanding of present obscurities.

One of the ultimate objectives of these studies was to prepare a rabies vaccine from cultures of tissues other than nerve cells. For this purpose, culture fluids of high infectivity are needed. The titers here recorded are probably not far below the requirements. It remains to work out a method of producing these culture fluids on a large scale.

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PREPARATION OF SPHEROPLASTS OF AEROBACTER CLOACAE[1]

B. S. W. SMITH,[2] JOHN I. PAYNE,[3] AND R. W. WATSON

Abstract

A method for the preparation of spheroplasts from *Aerobacter cloacae* has been devised. Washed cells are exposed to the combined action of lysozyme and 1.0 *M* tris buffer at pH 9.0 in 20% sucrose. Such treatment weakens the cell wall structure but most cells retain their rod shape. Subsequent dilution with three volumes of distilled water converts the rods instantaneously into spheres. The time of exposure to lysozyme required for satisfactory production of spheroplasts can be greatly reduced if the cells are preincubated with the tris buffer.

Introduction

Since the formation of protoplasts of *Bacillus megaterium* KM was reported by Weibull in 1953 (18) these osmotically fragile bodies have been the subject of much interest. It was soon realized that a valuable new technique for the production of metabolically active subcellular preparations had become available. This has lent impetus to the development of techniques for the preparation of similar bodies from an increasing number of species. Lederberg (9), and Zinder and Arndt (20), prepared 'protoplasts' from *Escherichia coli* by the action of penicillin and of lysozyme respectively, in the presence of a stabilizer, to record the first instances of the production of such bodies from Gram-negative organisms. Since this time 'protoplasts' or, as McQuillen (13) proposes to call them, "spheroplasts", have been prepared from several Gram-negative organisms (1, 3, 6, 12, 19).

During the course of an investigation into the significance of peptides present in extracts of *Aerobacter cloacae* it became desirable to utilize subcellular fractions of this organism. The modification of the procedure of Zinder and Arndt which was developed to convert *A. cloacae* into spheroplasts is described in this paper.

Materials and Methods

Growth of Cells

Stock cultures of *A. cloacae* NRC 492 were carried routinely on agar slopes containing 2% glucose, 0.5% yeast extract, and 1.5% agar (17), and stored in the refrigerator. A liquid medium (60 ml) containing 2% glucose, 0.6% $(NH_4)_2HPO_4$, 0.02% KH_2PO_4, and 0.025% $MgSO_4.7H_2O$ was inoculated from the stock culture and incubated at 30° C for 16 hours on a rotary shaker, then poured into 600 ml of the same medium and allowed to grow for a further

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6 hours. The optical density measured at 660 mμ on a Coleman model 6A spectrophotometer in a 10-mm cuvette was 0.41–0.43 (cell count 4.6–4.8 × 10^9 ml) (2).

Preparation of Spheroplasts

Cells grown as described above were centrifuged for 10 minutes at 17,000 *g* at 0° C, washed once in 0.85% saline, and resuspended in 100 ml of 1.0 *M* tris-hydroxymethylaminomethane (pH 9.0) containing 0.2% $MgSO_4.7H_2O$ and 20% sucrose. After 20 minutes at room temperature, 0.5 mg/ml of lysozyme (Nutritional Biochemicals Corp.) was added, and the suspension was allowed to stand for a further 10 minutes.

A sample of the suspension was added to three volumes of distilled water and examined under the phase microscope. If the yield were satisfactory the whole cell suspension was added to three times the volume of distilled water.

The extent of conversion to spheres was followed microscopically as described above, and also by measuring the change in optical density after lysis of the spheroplasts by a modification of the technique of Hurwitz *et al.* (5). One-milliliter samples of cell suspension were diluted with 3 ml of distilled water to form spheres. A further 3 ml of water at 56° C was then added to the diluted suspension and the whole heated at 56° C for 4 minutes to lyse the spheroplasts, the rods remaining intact. The reduction in optical density compared with control tubes, in which lysozyme was omitted, was measured at 660 mμ.

Experimental Results

Attempts to prepare spheres from *A. cloacae* by the Lederberg procedure (9), or by the action of lysozyme and versene as described by Repaske (14), were unsuccessful. However, lysozyme in 1.0 *M* tris buffer at pH 9.0 as used by Zinder and Arndt (20) gave a significant conversion to spheres. Cells grown in the glucose–salt medium were more susceptible to attack by lysozyme than were those harvested from Penassay broth, consequently we confined our subsequent experiments to the use of lysozyme in tris buffer at pH 9.0 on cells grown in glucose–salt medium.

Concentration of Lysozyme

Concentrations of lysozyme from 0.5 mg/ml to 2.0 mg/ml were found to be equally effective in the formation of spheres; the lower value was used routinely and the transformation to spheres was not affected by variations in cell density from 7.5–21 × 10^9 cells/ml.

Role of Tris Buffer

In the conversion of *E. coli* to spheroplasts Zinder and Arndt reported that tris buffer stabilized the spheres. This did not prove to be the case with *A. cloacae* and it was found necessary to add 20% sucrose to stabilize spheroplasts overnight.

Since sucrose was required as stabilizer it seemed possible that the molarity of the tris might well be reduced with advantage. However when 0.1 *M* buffer

at the same pH was used the rods aggregated and settled out in large clumps without formation of spheres.

The addition of tris-sucrose without lysozyme to cells generally caused a loss of motility, but even after 20 hours' exposure motility could be restored by dilution with water.

Microscopic examination of control tubes which contained tris but no lysozyme consistently showed a small number of spheres, indicating that the buffer had a significant effect on the cell wall in the absence of the enzyme; this is illustrated in Fig. 1.

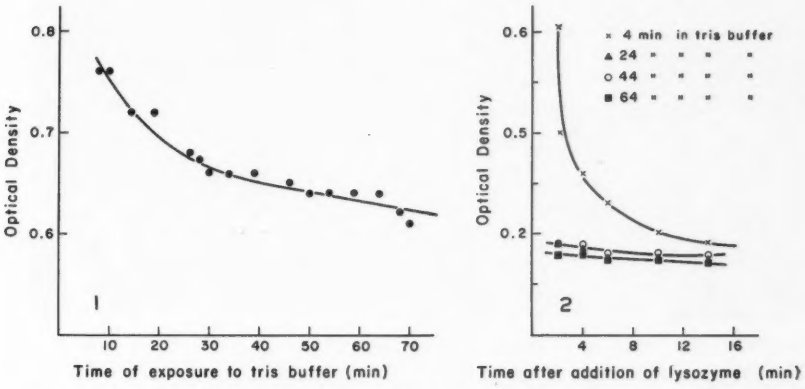


FIG. 1. Effect of time of incubation in tris buffer-sucrose on production of spheres. Optical density measured at 660 mμ after lysis of spheres by dilution and heating at 56° C.
FIG. 2. Effect of preincubation of cells in tris buffer (pH 9.0) and time of exposure to lysozyme on production of spheres. Optical density measured as in Fig. 1.

The effect of preincubation of cells with tris buffer on the time of exposure to lysozyme necessary to give quantitative formation of spheres was studied. Cells from a 600-ml culture were suspended in 50 ml of 1.0 *M* tris at pH 9.0 and allowed to stand at 25° C for specified periods before lysozyme was added. Conversion was determined by the lysis method. The results are shown in Fig. 2.

pH

Cells were incubated with lysozyme in 1.0 *M* tris at pH's varying from 7.0 to 10.5 in increments of 0.5; conversion was optimal from pH 8.5 to 9.5.

Extent of Dilution

The production of spheres was greatly increased by partial osmotic shock of the rods after incubation with lysozyme. In the early experiments dilution of the cell suspension with an equal volume of water was used routinely. Lysis was extensive overnight if the sucrose concentration was not restored to 20%. Conversion to spheres was maximal after $1\frac{1}{2}$–2 hours' incubation with lysozyme (added at the same time as the buffer).

In attempts to increase the yield of spheres after shorter periods of exposure to lysozyme we found that the conversion was progressively increased up to a dilution of one part of cells to three parts of water. At greater dilutions lysis was too rapid for the spheroplasts to be preserved. After dilution with three parts of water it was necessary to restore the concentration of stabilizer rapidly to 20% to keep the spheres intact; they were then stable overnight at room temperature. In 30% or 40% sucrose the spheres shrank to about one-half to one-third of their size at formation and appeared "crenated"; occasionally they formed crescents. The spheres could be centrifuged without lysis in 20% sucrose and were more stable at 5° C than at room temperature.

Age of Cells

Early experiments indicated that old cells in the stationary phase (24 hours) were more resistant to attack by lysozyme than were actively growing cells, but that spheroplasts formed from such old cells were more stable. During log phase growth no significant variation in the susceptibility to attack by lysozyme could be detected.

Microscopic Observations

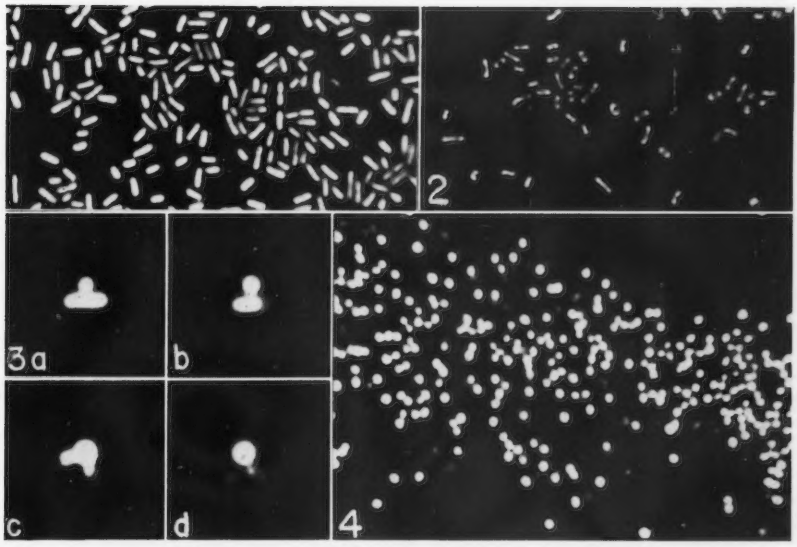
Cells in tris-sucrose often had retracted cytoplasm pulled away irregularly from the cell periphery (compare Fig. 3.2 with normal cells in growth medium, Fig. 3.1).

Intermediate forms which illustrate some of the stages in formation of spheres from rods can often be detected before dilution (Figs. 3.3*a*–3.3*d*). Bulges of various sizes may be seen (Fig. 3.3*a*), starting with a very small protrusion of the cell outline. These occur only at one place, usually centrally in the cell, but occasionally closer to one pole. The cytoplasm generally occupies the bulge as it enlarges, thereby forming characteristic "rabbits' ears" (Figs. 3.3*c* and 3.3*d*). Usually only a single sphere, a mass of which is shown in Fig. 3.4, is formed from each rod. Immediately after dilution, strands of material, presumably from the cell wall, adhering to the spheres could be seen under the microscope; these disappeared in a few minutes.

Discussion

The medium in which the organisms were grown had a pronounced influence on the ease with which spheroplasts could be produced. With *A. cloacae* cells grown in a synthetic glucose–salts medium were more easily converted to spheres than were cells harvested from Penassay broth. Schweighofer and Starlinger (16) have recently reported that in *E. coli* osmotically fragile rod-shaped "Halbprotoplasten" were obtained by growth in peptone water and were not evident in cells taken from a synthetic medium.

Microscopic examination of control samples containing tris buffer alone showed that the buffer had a small but significant effect on the cell wall structure which was manifested in the reduced time of exposure to lysozyme required after preincubation of the cells in the buffer. It appears that the bonds susceptible to attack by lysozyme were made more accessible to the enzyme.



FIGS. 3.1–3.4. Phase contrast photomicrographs of *Aerobacter cloacae*. Magnification, Figs. 1, 2, and 4 ca. ×1800; Figs. 3*a*–3*d* ca. ×3600. Fig. 3.1. Normal 6-hour cells in salts-glucose medium. Fig. 3.2. Six-hour cells in 1 *M* tris buffer (pH 9.0) and sucrose (20%). Figs. 3.3*a*–3.3*d*. Intermediate forms. Fig. 3.4. Spheroplasts in 20% sucrose

The necessity for dilution indicated that the lysozyme-tris combination was only able to weaken the wall structure to a limited extent. Microscopic examination immediately before dilution showed that only an occasional sphere was present, most of the cells remaining rod-shaped. This is in agreement with the recent report by Schweighofer and Starlinger (16) of the formation of rod-shaped "Halbprotoplasten" from *E. coli* by the action of lysozyme in 0.05 *M* tris and 0.5 *M* sucrose; these could be lysed by dilution with distilled water.

The formation of crescents in the presence of high concentrations of sucrose was occasionally observed. This is in accordance with the observations of Gebicki and James (3), who were able to transform spheroplasts of *Aerobacter aerogenes* into crescents by suspending them in media of higher osmotic strength. It seems clear that the formation of crescents is due to the presence of cell wall residue from which the cytoplasm is able to retract. With *A. cloacae* crescents could only be formed occasionally after dilution with an equal volume of water and never after dilution with three volumes of water, suggesting that this cell wall residue may be broken by the sudden swelling which takes place at the greater dilution.

The age of cells, provided they were in exponential growth, seemed to have no appreciable effect on the ease of spheroplast formation. This is in contrast to the recent work of Repaske (15), who reported a marked variation with age of cells in the lysability of *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, and *E. coli* by the action of lysozyme and versene in tris buffer at pH 8.

The various stages observed in spheroplast formation under the influence of lysozyme-tris are similar to those observed in other Gram-negative organisms exposed to the action of penicillin (4, 8, 10, 11). The electron microscopic pictures of *Proteus vulgaris* published by Liebermeister and Kellenberger (11) show very clearly prespheroplast forms similar to those observed with *A. cloacae*. These electron microscopic pictures show some cells with more than one bulge occurring per cell. Welsch and Osterrieth (19) also mention that sometimes two or more swellings occur in Gram-negative bacteria in the presence of penicillin. In the present study only one bulge was seen to occur per organism, although additional smaller bulges, beyond the limits of light microscopic resolution, may have occurred. Apparently one area in the inner, lipopolysaccharide layer of the cell wall, usually in the middle of the cell, is weaker or attacked more vigorously by lysozyme than the rest of the cell. Perhaps, since actively multiplying cells were used, this weak spot was somehow associated with the formation of a transverse septum. That this latter suggestion does not account for all mechanisms of spheroplast formation is exemplified by the method used with *E. coli* B in which freezing and thawing in the presence of lysozyme-sucrose caused multiple "tears and holes" in the outer cell wall layer, thereby exposing the inner layer to enzymatic action (7).

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CHANGES IN THE PHAGE-TYPING PATTERNS OF STAPHYLOCOCCI FOLLOWING LYSOGENIZATION WITH A RELATED GROUP OF STAPHYLOCOCCUS BACTERIOPHAGES[1]

R. D. COMTOIS

Abstract

Strains of phage type 52/52A/80 are appearing with increasing frequency in Canada, in conjunction with type '80/81' strains. To investigate the possibility of a relationship between these strains, attempts were made to lysogenize each of the propagating strains of phages 80, 81, 82, and KS6 with their homologous phages.

No lysogenic systems could be established with phage 80. The typing pattern of strains lysogenized with phages 81, 82, and KS6 differed from that of the parent strain. Three types of variants were observed. One type showed a loss in sensitivity to one or more of the phages. In the second, there was a concurrent loss in sensitivity to some of the phages and a gain in sensitivity to phages 52 and 52A; phages 81 and especially KS6 induced this type of variant. The third variant was either non-typable or typable only with other phages used undiluted. The change in phage sensitivity was not accompanied by a change in antibiotic sensitivity.

The possibility that strains of type 52/52A/80, as well as non-typable strains, may be derived in nature from type 80/81 strains by lysogenization with phage KS6, or one related to it, is discussed.

Introduction

Numerous epidemics caused by strains of phage type 80/81 staphylococci have been described in many countries in recent years (2, 3, 5, 6, 8, 11, 14, 15, 18). The strain was first reported in Australia in 1955 by Rountree and Freeman (11) as phage type 80, and independently in Canada in 1956 by Bynoe, Elder, and Comtois (5) as phage type 81.

During the last few years, an increasing number of strains lysed by phages 52 and 52A, in association with one or more of phages 80, 81, and 82, have been isolated in conjunction with strains of phage type 80/81 in hospitals in Canada. Asheshov and Rippon (1) reported that, in some prolonged epidemics caused by strains of phage type 80/81 staphylococci, strains of type 52/52A/80 were also isolated, particularly during the later part of the epidemic. Rountree (13) also reported instances of possible spontaneous mutation 'in vivo' of type 80/81 to type 52/52A/80/81 or type 52/80/81. Similar observations have also been made at this laboratory on the material submitted for typing.

The above observations therefore suggest that a close relationship exists between strains of phage type 80/81 and strains lysed by phages 52 and 52A in association with one or more of phages 80, 81, and 82. Evidence for this relationship was obtained by Asheshov and Rippon (1), who produced strains of phage types 52/52A/80, 52/52A/80/81, and 80, by lysogenizing 'in vitro' strains of type 80/81 staphylococci with phages derived from lysogenic strains

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of type 52/52A/80. Similar results were also reported by Rountree (13) following lysogenization of strains which showed a range of reactions with the phages 47C, 52, 52A, 80, and 81, with phages isolated from lysogenic strains of different phage types.

In the experiments described in this report, an attempt has been made to lysogenize a related group of staphylococci, namely, the propagating strains of phages 80, 81, 82, and KS6 (see below), with each of their homologous phages. It was thought that lysogenization of these strains might reveal further information concerning the origin in nature of strains of phage type 52/52A/80, 52/52A/80/81, or of other similar types.

Materials and Methods

Media

Trypticase soy broth (BBL) was used for broth cultures and trypticase soy agar (BBL) for typing, for propagation of phage, and for determination of lytic spectrum.

Phages

The phages used for typing were the 21 'basic' phages recommended by the Sub-Committee on Bacteriophage Typing of Staphylococci of the International Bacteriological Nomenclature Committee (20), together with phages 81, 82, and KS6. Twenty-two additional phages were also used to type the strains which were non-typable with the basic set.

Phages 81 and 82 were isolated by the author in 1953 and 1955, respectively, phage 81 by 'adaptation' of the classical phage 42B (5), and phage 82 by 'adaptation' of phage 52A, to new propagating strains; both are serological group A phages. Phage KS6 as well as its propagating strain (PS KS6) was received in October 1959 from Dr. Gösta Wallmark, State Bacteriological Laboratory, Stockholm, Sweden; it was isolated from a lysogenic strain of staphylococcus (17) by Fisk's cross-culture technique (7). Phage 80 used in the experiments to be described is a B phage serologically and was 'adapted' from phage 52A by Rountree in 1953 (11).

The phage stocks were prepared on agar by the freeze–thaw method of Williams and Rippon (19) and phages of satisfactory titers were Seitz-filtered and tested for sterility before use. For the experiments described below, phage filtrates with a routine test dilution (RTD) of not less than 1/1000 were used, the end point being the highest dilution just giving confluent lysis.

Cultures

The propagating strains used in this study were stock cultures in routine use at the National Staphylococcus Phage Typing Reference Centre, Laboratory of Hygiene, Ottawa, which had been received originally from the International Reference Laboratory, Colindale, England.

Determination of Lytic Spectrum of the Phages

The lytic spectrum of each phage was determined by the method of Williams and Rippon (19). The phage was first applied undiluted on a standard set of

propagating strains (Table I) and then titrated, using 10-fold dilutions, on those strains which were lysed. The phage titer on each strain lysed, relative to that on the homologous strain, was then calculated.

Lysogenization

Attempts were made to lysogenize in broth the propagating strains of phages 80 and 81 (PS 80 and PS 81) separately with each of phages 80, 81, 82, and KS6, and the propagating strains of phages 82 and KS6 (PS 82 and PS KS6) separately with each of phages 80, 82, and KS6. Phage 81 does not show lysis on PS 82 and PS KS6 and consequently was not used to lysogenize these two strains.

The strain to be lysogenized was grown in broth for 4–5 hours at 37° C and 2 ml was then mixed with an equal volume of the undiluted lysogenizing phage. After 2 days' incubation at 37° C the mixture was examined for turbidity and a loopful of the residual growth was subcultured onto an agar plate. After incubation, 10 single colonies were picked and subcultured 3 times in broth to eliminate transfer of phage from the original suspension. The last subculture was incubated for 4–5 hours at 37° C, and was then tested for resistance to the phage used for lysogenization and for any change in phage-typing pattern; it was also tested for the presence of the phage in the lysogenic state, by centrifuging for 15 minutes at 3000 r.p.m. and plating a loopful of the supernatant on agar previously seeded with the sensitive parent strain.

Coagulase Test

Three drops of an overnight broth culture were mixed with 0.5 ml of a freshly prepared 1/10 dilution of citrated rabbit plasma in saline. The mixture was incubated at 37° C for 4 hours and the results recorded.

Determination of Antibiotic Sensitivities

The antibiotic sensitivity tests were performed in the Biologics Control Laboratories of the Laboratory of Hygiene, by carefully standardized technical procedures advocated by this laboratory, using assayed disks (Difco) of 20 antibiotics of medium activity.

Results

Phages 80, 81, 82, and KS6 are all able to lyse at the RTD the majority of the 'classical' type '80/81' strains. During the last 3 years, of 798 strains received at this laboratory for typing and lysed exclusively by one or more of phages 80, 81, and 82, 455 were of phage type 80/81/82, 11 of type 80/81, 60 of type 80/82, 36 of type 81/82, 12 of type 80, 31 of type 81, and 193 of type 82. Since October 1959, phage KS6 has been used routinely in parallel with phages 80, 81, and 82, and has lysed all strains of type '80/81/82' submitted for typing during this period.

Lytic Spectrum of Phages 80, 81, 82, and KS6

The lytic spectrum of each of the phages 80, 81, 82, and KS6 used in the lysogenization experiments is compared in Table I. Each phage is lytically different, as shown by the wide variation of reactions on the strains tested.

Each of the phages gave titers on PS 80, PS 81, PS 82, and PS KS6 of equal strengths "–5–" to that obtained on its homologous propagating strain, with the exception of phage 81, which showed no titer on PS 82 and on PS KS6.

TABLE I

Lytic spectra of phages 80, 81, 82, and KS6

	Phage No.			
	80	81	82	KS6
	Relative titers*			
Test strain: PS 29	0	0	1	2
29A	.	4	.	3
42B/47C	2	5	5	5
42C	.	2	1	1
42D	.	1	.	1
42E	1	4	2	2
44A	3	2	1	3
47	1	.	.	.
51	.	.	.	.
52	5	.	1	1
53	.	1	1	.
54	.	3	1	4
80	5	5	5	5
81	5	5	5	5
82	5	.	5	5
KS6	5	.	5	5

*5 = maximum titer; 4 = $1/10-1/10^2$ of the maximum titer; 3 = $1/10^2-1/10^4$ of the maximum titer; 2 = $1/10^4-1/10^6$ of the maximum titer; 1 = occasional weak lysis; 0 = 'inhibition' with undiluted phage; . = no lysis seen.

Lysogenization

As mentioned under Materials and Methods, attempts were made to lysogenize in broth PS 80 and PS 81 separately with each of the phages 80, 81, 82, and KS6, and PS 82 and PS KS6 with phages 80, 82, and KS6. All mixtures of staphylococci and phage, with one exception, yielded dense growth after 2 days' incubation at 37° C; the residual growth of the mixture of PS 81 and phage 82 was poor. Complete lysis was not observed in any of the mixtures even after 7 days' incubation at 37° C. Ten colonies derived from each 'staphylococcus–phage' mixture after incubation were then phage typed and the results are shown in Table II.

Sensitivity to Lysogenizing Phage

It can be seen from Table II that the cultures from the colonies derived from the staphylococcus–phage mixtures were either sensitive (S), resistant (R), or partially resistant (R(S)) to the phage used for lysogenization when applied undiluted.

The colonies from the mixtures involving phage 80 yielded cultures which were all sensitive to phage 80; on the other hand, those derived from the mixtures containing phage 81 were all resistant to phage 81. The mixtures containing phage 82 yielded colonies some of which were sensitive and some resistant to phage 82; all colonies derived from PS 80, seven from PS 81, two from PS 82, and six from PS KS6 were sensitive, while two colonies from PS 81, seven from PS 82, and two from PS KS6 were resistant to phage 82.

TABLE II

Sensitivity to phage of colonies derived from PS 80, PS 81, PS 82, and PS KS6 following attempts at lysogenization

Strain No.	Phage pattern (RTD)	Lysogenizing phages	Sensitivity (S) or resistance (R) to lysogenizing phage	No. of colonies tested*	Phage pattern (RTD) following 'lysogenization'	Changes in phage sensitivity at RTD following lysogenization		Lysogenicity for parent strain
						Gains	Losses	
PS 80	80/81/82/KS6	80	S	10	80/81/82/KS6	—	—	Nil
		81	R	6	52/52A/80	52, 52A	81, 82, KS6	+
			R	2	Non-typable	—	80, 81, 82, KS6	+
		82	S	8	80/81/82/KS6	—	—	Nil
		KS6	R(S)	10	52/52A/80/81/82	52, 52A	KS6	+
PS 81	80/81/82/KS6	80	S	10	80/81/82/KS6	—	—	Nil
		81	R	10	80/KS6	—	81, 82	+
		82	S	7	80/81/82/KS6	—	—	Nil
			R	2	Non-typable	—	80, 81, 82, KS6	+
		KS6	R(S)	7	52/52A/80/81/82	52, 52A	KS6	+
			R	3	Non-typable (42B/47C/52B–1000 RTD)	—	80, 81, 82, KS6	+
PS 82	80/82/KS6	80	S	10	80/82/KS6	—	—	Nil
		82	R	7	Non-typable	—	80, 82, KS6	+
			S	2	80/82/KS6	—	—	Nil
		KS6	R	2	52/52A/80	52, 52A	82, KS6	+
			R	8	Non-typable	—	80, 82, KS6	+
PS KS6	52/52A/80/82/KS6	80	S	10	52/52A/80/82/KS6	—	—	Nil
		82	R	2	Non-typable	—	52, 52A, 80, 82, KS6	+
			S	6	52/52A/80/82/KS6	—	—	Nil
		KS6	R(S)	3	52/52A/80	—	82, KS6	+
			R	7	Non-typable	—	52, 52A, 80, 82, KS6	+

*Ten colonies were picked on agar but some failed to grow in broth.

The colonies from the mixtures involving phage KS6 were either resistant to phage KS6 (three colonies derived from PS 81, 10 from PS 82, and seven from PS KS6) or partially resistant (the 10 colonies derived from PS 80, seven from PS 81, and three from PS KS6). The partially resistant strains showed discrete plaques with only undiluted phage KS6 while the parent strains were lysed confluently by the RTD of this phage.

Phage Type or Pattern following 'Lysogenization'

From Table II, it can be seen that all colonies, the cultures of which were sensitive to the phage used for lysogenization, had the same phage pattern as the parent strains from which they were derived. The phage pattern of the colony, which was either resistant or partially resistant to the lysogenizing phage, differed, however, from that of the parent strain. These differences in the 'new' or 'variant' cultures took three forms. In one, there was a loss in sensitivity to one or more of the phages to which the parent strain was sensitive. The second type of variant showed, concurrently with a loss in sensitivity to some of the phages, a gain in sensitivity to phages 52 and 52A, to which the parent strain had been resistant. The third type of variant was either untypable or typable only with the additional phages used undiluted.

In PS 80, there was a change in pattern from 80/81/82/KS6 before lysogenization to 52/52A/80 in six of the colonies after lysogenization with phage 81 and to 52/52A/80/81/82 in all colonies lysogenized with phage KS6. Two colonies lysogenized with phage 81 became non-typable with all the phages used undiluted.

In PS 81, which has the same phage pattern as PS 80, it was expected that lysogenization would induce the same changes in phage sensitivity as those produced in PS 80. In all colonies lysogenized with phage 81, there was a change from 80/81/82/KS6 to 80/KS6; there was therefore a similar loss in sensitivity to phages 81 and 82 but there was no corresponding gain in sensitivity to phages 52 and 52A at the RTD. When tested, however, with undiluted phages, these 80/KS6 colonies gave plaques with phages 52 and 52A, whereas PS 81 before lysogenization showed only 'inhibition'. These variants, therefore, had become slightly sensitive to phages 52 and 52A. With phage 82, the effect of lysogenization was to produce in 2 of the 10 colonies tested complete immunity to all the phages, i.e. they became non-typable. Lysogenization with phage KS6 produced the same change in phage sensitivity in seven of the lysogenized colonies as it did in PS 80 (52/52A/80/81/82), while three colonies were typable only with the additional phages used undiluted.

In PS 82, there was a change from pattern 80/82/KS6 to 52/52A/80 in two colonies lysogenized with phage KS6 and immunity to all the phages in seven colonies lysogenized with phage 82 and in eight colonies lysogenized with phage KS6.

In PS KS6, two colonies lysogenized with phage 82, and seven colonies lysogenized with phage KS6 became non-typable, while in the other three colonies lysogenized with phage KS6, the sensitivity to phages 52, 52A, and 80 was retained but the sensitivity to phages 82 and KS6 was lost.

From these results it can be seen that the gain in sensitivity to phage following lysogenization was always one involving phages 52 and 52A, and was always accompanied by a loss in sensitivity to phage KS6 and, in some of the strains, to other phages as well.

Of the colonies that became non-typable with the basic set of phages following lysogenization, only three were typable with the 22 extra phages used undiluted. These three colonies were all derived from PS 81 lysogenized with phage KS6 and giving the pattern 42B/47C/52B. Since PS 81 before lysogenization was sensitive to these three phages, there was, therefore, no gain in phage sensitivity by these three variant cultures. All the non-typable colonies were pigmented and hemolytic on blood agar and were coagulase-positive; some of them had a tendency to grow with a granular deposit in broth.

Lysogenicity of the Colonies following Lysogenization

From the last column of Table II, it can be seen that all colonies which had acquired resistance to the phage used to lysogenize the parent culture and whose pattern had changed were found to be lysogenic for the parent strains from which they were derived. Conversely, sensitive colonies were not lysogenic for the parent strains.

Stability of Phage Types following Lysogenization

One colony representing each type shown in column 6 of Table II (21 cultures in all), whether sensitive or resistant to the lysogenizing phage, was subcultured daily in broth and was phage-typed daily for 10 days. A loopful from the last subculture of each strain was also plated on agar and five colonies were fished into broth and phage-typed.

All but two of the cultures showed the same results at each typing. In the lysogenized 'variant' strains, there was no indication of any reversion to the parent strain or of a change to a new phage pattern on subculture. The two cultures whose pattern was somewhat unstable on subculture were one culture of PS 81 lysogenized with phage KS6, non-typable with the basic set of phages but giving the pattern 42B/47C/52B, and one culture of PS 82 lysogenized with phage KS6 and giving the pattern 52/52A/80 at the RTD. In neither of these strains was there any reversal towards the original pattern.

In the first strain there was a change from pattern 42B/47C/52B to 52/52A/80/81/82 at the RTD on the fourth subculture in broth and this pattern was retained in all subsequent subcultures. From Table II, it can be seen that this is the pattern given by the other seven colonies of PS 81 lysogenized with phage KS6. The other two 'variant' colonies of type 42B/47C/52B, when later tested in the same way, were found to change also to 52/52A/80/81/82 on the fourth subculture. The change in pattern upon subculture of these three variant colonies was not accompanied by any loss in their sensitivity to phages 42B, 47C, or 52B.

In the other strain which showed some instability upon subculture in broth, there was a change from pattern 52/52A/80 to 52/52A/80/82 on the seventh subculture and this pattern was retained in all subsequent subcultures. The

TABLE III

Antibiotic sensitivities of PS 80, PS 81, PS 82, and PS KS6 before and after lysogenization

Strain No.	Phage used for lysogenization	Phage pattern before and after lysogenization	Antibiotics used: Bacitracin, Carbomycin, Chloramphenicol, Erythromycin, Kanamycin, Neomycin, Oleandomycin, Ristocetin, Soframycin, Spiromycin, Vancomycin	Streptomycin, Dihydrostreptomycin	Chlortetracycline, Oxytetracycline, Tetracycline	Novobiocin	Penicillin	Polymyxin, Viomycin
			Reactions before lysogenization					
PS 80		80/81/82/KS6	S*	S	S	R	R	R
PS 81		80/81/82/KS6	S	S	R	S	R	R
PS 82		80/82/KS6	S	R	R	S	R	R
PS KS6		52/52A/80/82/KS6	S	S	S	S	S	R
			Reactions after lysogenization					
PS 80	81	52/52A/80	S	S	S	R	R	R
PS 80	81	Non-typable	S	S	S	R	R	R
PS 80	KS6	52/52A/80/81/82	S	S	S	R	R	R
PS 81	81	80/KS6	S	S	R	S	R	R
PS 81	82	Non-typable	S	S	R	S	R	R
PS 81	KS6	52/52A/80/81/82	S	S	R	S	R	R
PS 81	KS6	42B/47C/52B	S	S	R	S	R	R
PS 82	82	Non-typable	S	R	R	S	R	R
PS 82	KS6	52/52A/80	S	R	R	S	R	R
PS 82	KS6	Non-typable	S	R	R	S	R	R
PS KS6	82	Non-typable	S	S	S	S	S	R
PS KS6	KS6	Non-typable	S	S	S	S	S	R
PS KS6	KS6	52/52A/80	S	S	S	S	S	R

*S = sensitive; R = resistant.

other culture of pattern 52/52A/80 derived from PS 82 lysogenized with phage KS6, when examined later in the same way, was also found to change to 52/52A/80/82 following subculture.

Antibiotic Sensitivities of the Lysogenized Strains

Colonies from each strain derived from PS 80, PS 81, PS 82, and PS KS6, and representing each phage type or pattern obtained following lysogenization, were tested for their sensitivities to 20 antibiotics using Difco disks of medium activity. The results were compared with the antibiotic sensitivities of the parent strains before lysogenization.

From Table III, it can be seen that no significant change in the antibiotic sensitivities of these strains occurred following lysogenization. In the lysogenized strains therefore, the change in phage type or pattern was not accompanied by a change in antibiotic sensitivity.

Discussion

The results reported in this paper confirm what other workers have found, namely, that the typing pattern of staphylococci can be changed in vitro as a result of lysogenization. Lowbury and Hood (9), following attempts to lysogenize the propagating strains with their homologous typing phages, reported that in the lysogenized strains there was a tendency for resistance to be acquired towards closely related phages. For instance, lysogenization of PS 75 with phage 75 produced a strain which was resistant to phages 75 and 75A; similarly a strain derived from PS 47 lysogenized with phage 47 was immune to phages 47, 47B, and 47C.

Since phages 80, 81, 82, and KS6 are all able to lyse the majority of type '80/81' strains and are therefore related to one another in this respect, it was anticipated that immunity to either one of these phages would be followed by immunity to the others. In the strains which became non-typable following lysogenization it is obvious that immunity occurred in blocks but not so in the typable strains, which were resistant to one or more but never to all four phages.

Asheshov and Rippon (1) reported changes in the typing pattern of type 80/81 strains following lysogenization with temperate phages isolated from lysogenic strains of type 52/52A/80 staphylococci. The changes were from phage type 80/81 to 52/52A/80, 52/52A/80/81, and 80, depending on the phages used for lysogenization. Four hypotheses to explain these changes—transduction, lysogenic conversion, selection of pre-existent mutants, prophage substitution—were discussed, but these authors claimed that their experimental results failed to support any of them. Rountree (13) in the same year also reported changes in the phage sensitivity of strains showing a range of reactions with the phages 47C, 52, 52A, 80, and 81; the changes took the form of either a loss or a gain (or both) in sensitivity to one or more of these phages following artificial lysogenization with lysogenic phages isolated from strains of phage types 80, 81, and 52/52A/80/81 staphylococci. She explained these changes as possibly due to prophage immunity or to the selection of spontaneous mutants by the converting phages.

In the work here reported, the typing pattern of strains which typed as 80/81/82/KS6 (PS 80 and PS 81), 80/82/KS6 (PS 82), and 52/52A/80/82/KS6 (PS KS6) was changed following lysogenization with one or more of phages 81, 82, and KS6; some of the lysogenized clones became immune to all the phages to which the parent strain was sensitive. Our failure to obtain a strain which was lysogenized with phage 80 confirms the results of Rountree (13), who also reported failure to set up lysogenic systems with this phage. The phage, therefore, appears to be a virulent mutant of phage 52A from which it was 'adapted'. The results indicate that the patterns produced in the laboratory by artificial lysogenization depend on the original phage sensitivity of the strain and on the phage used to lysogenize it. Thus, a strain of type 80/81/82/KS6 lysogenized with phage 81 became type 52/52A/80, but the same type was produced with an 80/82/KS6 or a 52/52A/80/82/KS6 strain lysogenized with phage KS6.

The number of non-typable strains derived from previously phage-sensitive strains and produced artificially in this study strongly suggests that lysogenization plays an important role in the origin of non-typable strains in nature. There seems to be some evidence for this in the specimens submitted for typing to our National Reference Typing Centre. For instance, very frequently two strains, one of type 80/81 and the other non-typable but giving inhibition with phages 80, 81, or 82, have been received from one patient. It is possible that the patient was originally infected with an 80/81 strain which later became lysogenized with a phage conferring immunity to all the typing phages. How frequent such changes in type or pattern may occur in nature is not known, but there is no reason why they should not occur.

Of particular interest was the gain in sensitivity to phages 52 and 52A and the concurrent loss in sensitivity to phage KS6 in PS 80, PS 81, and PS 82 lysogenized with phage KS6 and in PS 80 lysogenized with phage 81. The propagating strain of phage KS6 is lysed by phages 52 and 52A at the RTD. Therefore in the colonies lysogenized with phage KS6, the cultures of which were sensitive to phages 52 and 52A, it would appear that lysogenization confers some of the characteristics of the strain on which phage KS6 is propagated. The loss in sensitivity to phage KS6 in the strains lysogenized with this phage indicates immunity in Lwoff's sense (10) but the gain in sensitivity to phages 52 and 52A is more difficult to explain. Gain in sensitivity to these phages has also been shown by Asheshov and Rippon (1) and by Rountree (13) in the work referred to above. Gain in sensitivity to phage following lysogenization has also been reported in other species of bacteria, namely, in *Salmonella anatum* (16) and in *Salmonella paratyphi B* (4).

Rountree (12) reported that immunity following artificial lysogenization was serologically specific in that lysogenization with an A phage conferred immunity to other A phages but not to B phages and vice versa. In her later publication (13), an example was given of this serological specificity of prophage immunity, but she also showed that this was not a universal phenomenon since she found that the prophage of an F phage immunized against an A phage. The results reported in this paper confirm her later findings. Phage 81,

a group A phage serologically, has been shown to immunize against phage 82, also group A and vice versa. On the other hand, phage 81 has also been found to immunize against phage 80, a group B phage serologically, and phage 82 against phages 52, 52A, and 80, all serological group B phages. Phage KS6 was found in this laboratory to be also a serological group A phage and has also been shown to immunize against both serological group A and B phages.

Of epidemiological interest was the observation that alteration in phage sensitivity by lysogenization does not necessarily alter the antibiotic spectrum of the lysogenized strains. This finding further demonstrates the severe limitation of sensitivity to antibiotics as a means of typing staphylococci in epidemiological studies. Furthermore whatever mechanism was responsible for the gain in sensitivity to phages 52 and 52A in the strains lysogenized with phage KS6, it did not alter their reactions to penicillin and the tetracyclines to which the propagating strain of phage KS6 is sensitive.

The observation that strains of phage type 52/52A/80 (52/52A/80/81/82, or other combinations of 80, 81, and 82 with phages 52 and 52A) were appearing with increasing frequency in Canada, led us to investigate the possibility of some relationship existing between these types and the classical type 80/81, which since 1955 had been the predominant type in 'hospital' infections in Canada. We have successfully demonstrated that these types (as well as non-typable strains) can be produced in vitro by lysogenization of '80/81' strains with a related group of phages. Phage KS6 was especially able to induce this change. Since phage KS6 was originally isolated from a lysogenic strain of staphylococcus and strains lysed exclusively by it have been isolated in Canada, it is at least possible that such a phage, or one related to it, could produce similar changes in strains in nature. This also suggests the interesting possibility that following outbreaks of '80/81' infections in hospitals, the epidemic strains become more and more exposed to association with other strains. If this association includes lysogenic strains of closely related types, the typical 80/81 strain may be changed by lysogenization to a 52/52A/80 or similar type. This is not to suggest that the new 52/52A/80 strain is any less virulent than the parent 80/81 strain. Gain or loss in sensitivity to phage does not seem to be in any way related to virulence.

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EVIDENCE FOR THE EXISTENCE OF ACID-SENSITIVE ACTINOMYCETES IN SOIL[1]

W. A. TABER

Abstract

A total of 1269 actinomycetes was isolated from 10 alkaline and 6 acid Canadian soils. One hundred and seven were unable to grow on a dilute glucose–asparagine–salts agar which had been adjusted to pH 6.2, but were able to grow when the pH was adjusted to 8.5. Additional tests of 24 of the isolates showed that the lowest pH's allowing growth ranged from 6.8 to 6.1, the actual value varying with the isolate and with the composition of the medium. These isolates required a higher pH for growth on all media tested than did a typical *Streptomyces* which was included in the study to serve as a control. None was as sensitive to acidity, however, as the alkaline-dependent *Streptomyces caeruleus* Baldacci.

The 107 isolates were interpreted to represent no more than 14 distinct organisms and 6 of these bore aerial hyphae and spores in chains. Nine of the 14 were some shade of orange or purple and the remainder were some shade of blue. A blue, sporulating *Streptomyces* (PRL 1772) resembled closely *S. caeruleus*.

Acid-sensitive actinomycetes were found in 9 of the 10 alkaline soils and in 1 of the 6 acid soils.

Four samples of beach mud from an alkaline salt lake yielded but one species of *Streptomyces* and it was sensitive to acidity. Its tolerance to sodium chloride was but slightly greater than that of a *Streptomyces* judged to be conventional in its response to acidity.

Introduction

Actinomycetes grow best in neutral or alkaline media (1), and at one time were thought to require an alkaline reaction for growth but this view is no longer held (12). The isolation of the alkaline-dependent *Streptomyces caeruleus* Baldacci (3, 8) from this region of alkaline soils suggested that an acid-sensitive actinomycete flora might exist. This hypothesis was tested by isolating actinomycetes from alkaline and acid soils and by comparing their ability to grow at pH 6.2 and 8.5. An acid-sensitive actinomycete is arbitrarily defined as one which requires a pH of 6.2 or more for growth when using a medium consisting of glucose–L-asparagine–salts and agar. That this end point is sufficiently critical is attested to by the fact that only 8% of the isolates failed to grow at pH 6.2.

This paper reports the isolation of acid-sensitive actinomycetes from alkaline soil, demonstrates that the lower pH limit varies among them, and provides evidence that they comprise but a small proportion of the actinomycete flora.

Materials and Methods

Plating Procedure

Approximately 10 g of soil was collected from each station (Table I) and was air-dried in sterile petri plates. Each soil sample was plated by suspending 1 g in 99 ml of sterile distilled water, shaking the suspension 50 times, and continuing the dilutions serially in sterile distilled water in the usual way.

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Approximately 12 plates were poured at 10^{-4} and at 10^{-5} dilutions. To three-fourths of the plates in each set was added, at the time of preparation, 0.5 mg of candidin (11) which had been dissolved in 0.5 ml of 0.01 *N* NaOH and sterilized by filtration.

A comparison of the actinomycete counts of plates which contained candidin with those which did not showed that this polyene antifungal antibiotic did not suppress the actinomycete count, and, indeed, few plates that did not contain candidin were sufficiently free of fungi to permit the isolation of actinomycetes. Soils having a low actinomycete count were replated at 10^{-2} and 10^{-3} dilutions. The dilute alkaline plating medium (2B) is described below. The plates were incubated 17 days at 28° C and then a total of 80 isolations were made from each set of plates. A special effort was made to isolate from pinpoint colonies which did not bear dry aerial hyphae since this type of actinomycete might be overlooked by routine surveys. With this exception, an attempt was made to select colonies at random from the plates for isolation. The isolations were made by transferring a portion of each selected colony onto slants of the alkaline plating medium (2B) and onto slants of acid medium of the same basic composition but with the phosphate buffer concentration modified. Those cultures growing only on the alkaline medium were transferred to fresh slants of the alkaline and acid medium. When all of the soils had been plated, those isolates still showing acid-sensitivity were simultaneously tested on additional media.

TABLE I

Origin of soil samples

Soil number, in order of plating	Source		pH
1A	Sudbury, Ontario	Grass covered lot, railroad station	6.2
1B	Sudbury, Ontario	Grass covered lot, railroad station	6.2
2	Macdonald College Farm, Quebec	Deciduous forest	6.8
3	Saskatoon, Saskatchewan	Wheat field north of city	7.5
4	Macdonald College Farm, Quebec	Alfalfa field	6.5
5	Saskatoon, Saskatchewan	Potato plot north of city	7.7
6	Saskatoon, Saskatchewan	Soil beneath club moss, Beaver Creek hill	6.7
7	Saskatoon, Saskatchewan	Soil beneath prickly pear, base of Beaver Creek Hill	7.1
8	Cudworth, Saskatchewan	Freshly cut down deciduous forest	7.0
9	Cudworth, Saskatchewan	Beach mud of Muskiki (salt) Lake	8.7
10	Cudworth, Saskatchewan	Pasture 100 yards from beach	7.9
11	Saskatoon, Saskatchewan	Barren sand bank, Beaver Creek	8.0
12	Guelph, Ontario	Guelph loam	7.5
13	Guelph, Ontario	Fox loam	7.5
14	Macdonald College Farm, Quebec	Cucurbit field	7.6
15	Macdonald College Farm, Quebec	Alfalfa field	6.5

Soil pH

The pH of the soil was measured with a Beckman glass electrode pH meter. The soil suspension was prepared by suspending 1 g of soil in 5 ml of distilled water (7). The reading was taken after the suspension had stood for at least 1 hour.

Media

Dilute Synthetic Media

	Medium 2B	Medium 2C
Fisher anhydrous dextrose	1 g	1 g
NBC L-asparagine	0.5 g	0.5 g
K_2HPO_4	0.750 g	0.400 g
KH_2PO_4	None	0.500 g
Agar	15 g	15 g
Distilled water to:	1 liter	1 liter

The pH of medium 2B was adjusted to 8.5–8.7 with 20% KOH before the addition of agar. The final pH of 2B after autoclaving was 8.4, and of 2C, 6.2.

Rich Synthetic Media (2A Series)

The basal medium consisted of the following:

Fisher anhydrous dextrose	26 g
NBC L-asparagine	3 g
$MgSO_4.7H_2O$	202 mg
$FeSO_4.7H_2O$	5 mg
$CaCl_2.2H_2O$	5.5 mg
$ZnSO_4.7H_2O$	4.4 mg
$MnSO_4.H_2O$	2.75 mg
$CuCl_2.2H_2O$	0.269 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.82 mg
NaCl	2.55 mg
Agar	15 g
Distilled water to:	1 liter

The glucose was autoclaved separately. Media of various pH's were prepared by adding different proportions of phosphate salts during preparation as follows:

Medium	K_2HPO_4/liter	KH_2PO_4/liter
2A — pH 8.5	3 g	None
2A — pH 6.9	1.5 g	1.0 g
2A — pH 6.7	1.2 g	1.3 g
2A — pH 6.6	1.1 g	1.4 g
2A — pH 6.3	0.9 g	1.6 g
2A — pH 6.1	0.6 g	1.9 g

Medium 2A—pH 8.5 was brought to pH 8.5–8.7 by adding sufficient 20% KOH to the basal medium. Liquid media to be used for growth in shaken culture were prepared by omitting agar.

Potato Plug Media

The descriptions of actinomycetes are commonly based, in part, upon their appearance when grown on potato plugs; therefore an inability to grow on potato plugs, unless they are adjusted to a high pH, could be taken as evidence for an unusual sensitivity to acidity.

Plugs of new potato were cut with a cork borer and placed in test tubes which were then plugged with cotton and sterilized. After cooling, sufficient sterile distilled water was added to cover all but the surface of the plug. The pH of the water after 1 week was 5.6. Alkaline potato plugs were prepared by adding 10% K_2HPO_4 to sterile plugs. The pH after 1 week was approximately 8.0.

Complex Organic Media (WGA (12))

Fisher anhydrous dextrose	10 g
Difco bacto peptone	5 g
Difco beef extract	5 g
NaCl	5 g
Distilled water to:	1 liter
Adjust pH 7.2 with KOH	
Agar	15 g

Flasks of the completed sterile agar were melted in a boiling water bath and sufficient KOH or H_2SO_4 added to bring the pH to one of the following values: 8.0, 6.9, 6.2, 5.5, or 5.1. Six milliliters of sterile medium were dispensed per sterile, dry test tube.

Streptomyces Reference Cultures

The acid-sensitivity of a fresh isolate could best be demonstrated by comparing its growth with both a typical *Streptomyces* and a *Streptomyces* already established as being unusually sensitive to acid. *Streptomyces* sp. PRL 1642, which produces amidomycin (10) and valinomycin, was selected to represent a "typical" *Streptomyces* because it is capable of rapid growth on media commonly used to grow *Streptomyces*. *Streptomyces caeruleus* Baldacci (PRL 1687) was used to represent acid-sensitive actinomycetes because it is the only one reported in the literature.

Inoculum

For Shaken Cultures

Cultures were grown with shaking at 30° C in flasks of 2A—pH 8.5 liquid media. As soon as the growth had become abundant the mycelia were washed twice with sterile physiological saline (.85%) and then suspended in sufficient saline to give a concentration of mycelium of approximately 0.1 mg/0.1 ml, dry weight equivalent. Each flask of experimental medium was inoculated with 0.1 ml of suspension.

For Solid Media

Small fragments of mycelia were removed from test tube cultures grown on 2A—pH 8.5 agar and were used to inoculate tubes or plates of experimental media.

For Manometric Studies

Aliquots of actively growing shaken cultures grown on 2A—pH 8.5 or YD—pH 7.5 (1% glucose–1% yeast extract and adjusted to pH 7.5) media were centrifuged, decanted, and washed three times with either pH 6.2 or pH 7.8 *M*/15 phosphate buffer. The washed mycelia were suspended in the appropriate buffer to give concentrations of mycelia ranging from 3–8 mg, dry weight equivalent.

Manometric Studies

Five of the acid-sensitive cultures and the two reference cultures were tested for their ability to respire exogenous glucose at pH 6.2 and 7.8. The tests were carried out in conventional Warburg vessels. Each vessel contained 1 ml of mycelial suspension, 1.5 ml buffer, 25 μmoles glucose in 0.5 ml buffer, and

0.2 ml 20% KOH in the well. The vessels were incubated at 30° C. The Qo_2 was computed from the difference between oxygen uptake in the presence and in the absence of exogenous glucose. While this procedure is frequently used, there is some reason to believe that it is not always necessary (6).

Antagonistic Activity

The actinomycetes were each streaked onto 20 ml of YD—pH 7.5 agar contained in a petri plate, and the plates were then incubated at 28° C until growth was heavy. Suspensions of *Candida albicans* (ATCC 10231), *Staphylococcus aureus* (PRL M2), *Escherichia coli* (PRL R2), and *Bacillus subtilis* (B44) were then streaked at right angles to the actinomycete colony. After 24 and 48 hours the zone of inhibition of growth was measured.

Results

Demonstration of Acid-sensitivity

Growth on Dilute Synthetic and Potato Solid Media

One hundred and seven of the 1269 isolates failed to grow at pH 6.2 (medium 2C) but grew abundantly at pH 8.5 (medium 2B). Eighty of the 107 isolates came from one soil and appeared to be identical, and therefore one was selected to represent the group for further studies. Four of the 27 remaining acid-sensitive isolates grew poorly on transfer and were discarded. A total of 24 isolates was studied further.

The 24 isolates were again transferred onto the alkaline and acid media and again growth occurred only on the alkaline medium. These 24 isolates, then, conformed to the definition of acid-sensitivity set forth in the Introduction of this paper. In order to determine whether acid-sensitivity was demonstrable only on the basal medium of media 2B and 2C, these isolates were inoculated onto media of different compositions and pH's and the presence or absence of growth was noted.

When they were inoculated onto both the acid (unadjusted) and alkaline potato plugs, 14 grew only on the alkaline plugs, 3 grew on acid and alkaline plugs, and 7 grew so poorly that a comparison could not be made. The latter were subsequently found to grow slowly on all of the media that were used in the study. The fact that most of these isolates would not grow on a medium routinely used in the study of actinomycetes, unless it was adjusted to a higher pH, testifies to their unusual sensitivity to acidity. The three that grew on acid and alkaline potato plugs were, however, acid-sensitive as judged by growth on the various other media of different pH's.

Growth on Rich Synthetic Agar Media

The 24 isolates were next inoculated onto the rich synthetic solid media of various pH's. Relative growth of representative strains is reported in Table II. Not one of the isolates grew into the agar adjusted to pH 6.1 and only two grew into the agar adjusted to pH 6.3. The control representing typical *Streptomyces* grew as well at pH 6.1 as at any of the higher pH's. The sensitivity to acidity, then, is not associated only with dilute media or with potato plugs.

TABLE II

Growth on rich synthetic agar media* (10 days)

Strain	pH					
	8.5	6.9	6.7	6.6	6.3	6.1
Control PRL 1642	4+	4+	4+	4+	4+	4+
Control PRL 1687	4+	0	0	0	0	0
7– 7	2+	3+	3+	3+	3+	0
6–24	3+	3+	2+	2+	2+	0
10–42	3+	3+	2+	2+	1+	0
9–37	4+	3+	2+	2+	1+	0
10–66	4+	2+	0	0	0	0
13–23	2+	3+	3+	2+	0	0
14–31	3+	3+	2+	2+	0	0
14–66	2+	1+	0	0	0	0
13–18	3+	2+	2+	1+	1+	1+
11–33	3+	3+	3+	3+	1+	0
11–16	2+	3+	3+	3+	2+	1+
12–39	2+	2+	1+	1+	1+	0
12–70	3+	3+	2+	2+	1+	1+

*All 24 strains were tested for growth on these media but only representative members of the group are referred to in this table.

NOTE: 4+ = growth covering most of agar surface.
3+ = growth extending beyond inoculation streak.
2+ = growth limited to area of inoculation streak.
1+ = growth limited to slight expansion of inoculum, but with no growth on agar.
0 = no growth.

Growth on Rich Liquid Synthetic Media

The isolates were next grown on the liquid synthetic media of varying pH (Table III). The strains which were previously found to grow poorly on potato plugs and rich synthetic agar media did not produce sufficient yields to serve as inocula, hence they were not included in this study.

None of the strains was able to grow readily on the medium adjusted to pH 6.1, but all could grow when inoculated into media of higher pH. Strain 10-66*b* required a pH of at least 6.8 to grow while the other strains required a pH of at least 6.2 for growth. Note that the culture intended to represent typical *Streptomyces* grew as well at pH 6.1 as at any higher pH, except 8.5, and that the acid-sensitive *S. caeruleus* did not grow at pH 6.9 or less.

Growth on Complex Organic Agar Media

When the 24 isolates and controls were inoculated onto the complex organic media of various pH's, the typical *Streptomyces* grew as well at pH 5.5 as at higher pH's, but only two of the 24 acid-sensitive strains grew at pH 5.5 and only 5 grew at pH 6.1. The inoculum of *S. caeruleus* expanded slightly but did not grow into the agar of pH 6.9 or less. These data suggest that although the lowest pH permitting growth varies slightly with the composition of the medium, the requirement for a high pH cannot be replaced by the complex organic nutrients present in basal medium WGA. None of the cultures grew on the agar adjusted to pH 5.0.

Respiration

The low pH might prevent growth by any one of several mechanisms. The fact that at low pH inocula can expand slightly but do not lead to continued growth suggested that while some nutrients were accessible to the cells,

TABLE III

Growth on rich synthetic media in shaken culture (mg dry weight per flask culture*)

		Initial pH											
		2A—pH 8.5		2A—pH 6.9		2A—pH 6.7		2A—pH 6.6		2A—pH 6.3		2A—pH 6.1	
Culture	Days	Dry weight	Final pH	Dry weight	Final pH	Dry weight	Final pH	Dry weight	Final pH	Dry weight	Final pH	Dry weight	Final pH
PRL 1642	7	318.2 ± 5	6.7	226.7 ± 2.6	5.4	223.7 ± 19.1	5.5	224.5 ± 37	5.1	202.8 ± 18	5.3	217.1 ± 8	6.1
PRL 1687	7	189.7 ± 35.1	8.0	0	6.9	0	6.7	0	6.6	0	6.3	0	6.1
6–24	7	187.2 ± 5.0	8.3	143.9 ± 5.0	6.8	14.0 ± 6.3	6.7	3.0 ± 2.0	6.6	1.6 ± 0.1	6.5	0	6.3
7–72	8	29.5 ± 3.0	8.8	130.6 ± 5.4	8.3	52.4 ± 3.1	7.3	8.9 ± 0.6	7.0	0	6.3	0	6.1
10–41	8	91.7 ± 4.0	4.9	45.4 ± 4.0	6.3	22.1 ± 1	5.9	10.0 ± 0.5	5.4	5.1 ± 0.3	4.8	2.1 ± 0.2	4.9
9–37	10	93.9 ± 2.2	8.1	29.5 ± 1.0	6.5	23.1 ± 1.0	6.5	26.5 ± 1.5	6.5	31.1 ± 7.0	6.0	0	6.1
7–7	10	8.9 ± 0.4	8.1	147.9 ± 18.1	7.9	37.1 ± 4.0	7.2	2.1 ± 0.1	6.7	24.3 ± 2.0	6.7	0	6.2
7–15	10	9.4 ± 1.3	8.3	94.3 ± 8.1	8.2	20.3 ± 0.1	7.0	24.7 ± 0.1	6.9	5.8 ± 2.0	6.6	0	6.1
10–43	3	106.0 ± 1.0	4.7	31.4 ± 3.0	6.3	25.4 ± 7.0	6.2	6.6 ± 0.2	6.1	5.6 ± 0.5	5.2	2.6 ± 0.3	4.9
10–9	4	145.2 ± 7.1	8.2	76.8 ± 1.0	6.3	18.6 ± 0.6	5.8	15.7 ± 0.3	5.9	4.5 ± 2.2	5.0	4.1 ± 1.2	4.7
7–48	10	3.7 ± 1.1	8.0	84.2 ± 2.1	7.9	8.8 ± 1.2	7.3	14.9 ± 0.5	7.3	7.8 ± 2.5	6.7	0	6.2
10–66*b*	8	115.1 ± 33.1	8.3	6.9 ± 1.2	6.9	0	6.7	0	6.6	0	6.4	0	6.2
11–33	7	219.5 ± 3.7	8.3	46.4 ± 1.0	7.0	28.9 ± 8.1	6.8	4.9 ± 0.1	6.6	1.6 ± 0.1	6.4	1.8 ± 0.1	6.2
13–23	11	51.6 ± 3.8	7.8	37.9 ± 10.1	6.8	3.1 ± 0.6	6.7	1.8 ± 0.1	6.6	2.4 ± 0.6	6.2	0	6.1
12–39	10	27.5 ± 7.0	6.6	4.7 ± 0.7	6.5	3.2 ± 0.2	6.5	2.7 ± 0.2	6.5	2.7 ± 0.1	6.3	3.0 ± 0.1	6.1

*A dry weight value less than 1 mg per flask culture was considered insignificant. The values are the arithmetic mean and standard error of three replicate cultures (9).

others were not and thus after exhaustion of reserves growth ceased. If the assimilation of glucose were prevented by the low pH, this effect might be reflected in the inability of cells to respire exogenous glucose. All of the isolates that were examined (Table IV) were able to oxidize exogenous glucose at pH 6.2, and thus no evidence was obtained to support the above hypothesis. Only *S. caeruleus* respired glucose more rapidly at the higher pH.

TABLE IV

Respiration in buffer at pH 6.2 and 7.8

Strain	Mycelium grown on:	Q_{O_2} glucose		Endogenous (μl O_2/mg/hour)	
		pH 6.2	pH 7.8	pH 6.2	pH 7.8
Control PRL 1642	YD—pH 7.5*	15.7	9.5	9.5	17.4
Control PRL 1642	*2A—pH 8.5†*	*10.4*	*3.9*	*5.4*	*6.9*
PRL 1687	2A—pH 8.5	3.2	10.7	16.4	12.3
6–24	2A—pH 8.5	6.7	2.6	21.3	23.9
7–15	YD—pH 7.5	9.9	9.1	17.4	19.7
10–66b	2A—pH 8.5	*29*	*26*	11	8.9
13–23	YD—pH 7.5	4.1	0	6.5	7.9
9–37	YD—pH 7.5	10.8	4.1	6.9	8.9

*1% glucose – 1% yeast extract, adjusted to pH 7.5 after sterilization.
†Glucose–asparagine medium adjusted to pH 8.5.

Antagonistic Activity

Krassilnikov (5) considers the production of antibiotics by actinomycetes to be of species significance. Twenty-one isolates were tested for inhibition of *Candida albicans*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* to determine whether they were separable on the basis of antagonistic activity.

The 21 isolates that were assayed clearly differed with respect to antagonistic activity. Twelve of the isolates, representing 8 of the 14 final groupings (see Table V), inhibited one or more of the test organisms. Isolate 10-66*a*,*b* resembles *S. caeruleus* quite closely in morphology and pigmentation, and also in the microorganisms that it antagonizes. Representatives of 4 of the final 14 groupings inhibited *S. aureus* and *C. albicans*. None inhibited *E. coli*.

If Krassilnikov's premise that antibiotic production is a species characteristic is correct, then the 14 groupings given in Table V do represent more than one species.

Description of Cultures

Those isolates which were obtained from the same plate and which appeared to be identical were tentatively considered to be single species; the 24 isolates were then reduced to 14 cultures and of these a number appeared similar (Table V). There was not sufficient basis for grouping similar cultures from different soils together, however; hence these were kept separate and were assigned different culture collection numbers.

None of the 14 was recognizable as a known species although the orange non-sporulating ones resemble *Nocardia* closely. It may prove to be of significance taxonomically that all of the acid-sensitive cultures were some shade

TABLE V

Description of cultures (30° C; 2A—pH 8.5 agar)

PRL	Strain No.	Petri plate cultures, 20 ml agar
1767	7–7; 7–15; 7–48; 7–72	Bright orange (12.5, 2.5 YR, 6/12*) wrinkled, raised moist mat. No aerial hyphae. Reverse orange. No diffusing pigment
1762	6–24	Deep orange (10, 10R, 4/10), becoming reddish orange (7.5, 7.5R, 3/10). Raised, wrinkled moist mat. No aerial hyphae. Reverse deep orange. No diffusing pigment
1777	10–42; 10–52	Small pinpoint green-black (50, 10.6, 2/2) moist, smooth raised mat. Eventually supports thin layer of slate blue aerial hyphae. Reverse dark green. Green diffusing pigment. On WGA—pH 7.5, mat is dark brown
1764	10–41; 10–43; 10–45; 10–9	Spore mass white, then becoming sky gray blue (67.5, 7.5B, 3/2). Spore chain straight or flexuous and branched. Spores globose and subglobose 1.2–1.4 × 1.1 μ. Reverse brown. No diffusing pigment
1763	9–37	Raised yellow mat eventually supporting cream aerial hyphae and spores. Spore chain long and flexuous. Spore oblong to cylindrical, 1.7–2.2 × .7 μ. Spore mass of 20-day-old WGA grown cultures became pale blue. Reverse orange brown. No diffusing pigment
1772	10–66*a*; 10–66*b*	Spore mass at first cream, becoming pale purple on WGA—pH 7.5 agar. Spore mass is purple (85, 5.P, 7/2) after 15 days' incubation. Spore chain straight and short. Spore cylindrical 1.3–3 × 0.7 μ. Reverse brown, becoming blue under conditions favoring blue pigmentation of spores
1775	12–70	Bright orange (12.5, 2.5 YR, 6/12) rough, raised, moist mat. No aerial hyphae. Reverse orange. No diffusing pigment
1770	12–39	Deep orange (10, 10R, 4/10) raised moist mat. Occasionally supporting sparse white aerial hyphae. Reverse orange. Trace of diffusing orange pigment
1778	11–16	Bright orange (12.5, 2.5 YR, 6/12) raised, moist mat. No aerial hyphae. May be same as 12–70. Reverse orange. No diffusing pigment
1776	11–33	Purple (10, 10R, 4/10) raised, rough, moist mat. No aerial hyphae. Reverse purple orange. No diffusing pigment. On WGA—pH 7.5 agar colony becomes brownish-orange
1773	13–18	Bright orange (12.5, 2.5 YR, 6/12) raised moist mat. Reverse orange. No diffusing pigment. May be same as 11–16
1774	13–23; 13–39	Purple orange (10, 10R, 4/10) raised moist mat. No aerial hyphae. Reverse orange. No diffusing pigment. Pigment of cultures grown on WGA—pH 7.5 is bright orange
1784	14–31; 14–28	White mat supporting sparse white aerial hyphae, later pale blue. Reverse white. No diffusing pigment
1785	14–66	Bright orange (12.5, 2.5 YR, 6/12) wrinkled, raised moist mat. No aerial hyphae. Reverse orange

*Munsell Book of Color.

TABLE VI

Some characteristics of the actinomycete population of 16 soil samples

Soil sample	pH	Lavender-gray	Griseus green	Pink-orange	White cream	Buff	Blue-purple	Black	Brown (moist)	Colonies per gram soil	Number acid-sensitive strains
1A	6.2	30	4	5	41					332,000	0
1B	6.2	15	3	7	43	2	2	2	8	165,000	0
2	6.8	62	3	2	11					2,050,000	0
3	7.5	44	6	9	20		1			730,000	3†
4	6.5	67		5	4					1,100,000	0
5	7.7	20	6	14	38			2		730,000	2†
6	6.7	55	1	2	9		2	10		1,200,000	1
7	7.1	40	3	3	6			28		190,000	4
8	7.0*	48	4	7	18			3		2,050,000	0
9	8.7						80			39,700	80
10	7.9	28	1	7	10	17		17		1,270,000	7
11	8.0	22		16	35	5		1		228,000	2
12	7.5	34	1	3	40	1		1		11,000,000	2
13	7.5	47		7	22		2	2		2,040,000	3
14	7.6	48		5	24		1			146,000	3
15	6.5	49	3	3	18	3		1		1,5410,000	0
Total (1269 isolates)		609	35	97	339	26	88	67	8		107

*Classed as an alkaline soil.
†Not examined beyond the two tests on 2B and 2C media.

of blue, purple, or orange. This fact would suggest that the 14 isolates represent but a limited number of species which are widespread in alkaline soils. The acid-sensitive reference culture, *S. caeruleus* Baldacci, is blue and cultures PRL 1763 and PRL 1772 resemble this known species somewhat. Until species criteria which are widely accepted have been established for *Streptomyces*, however, there is no advantage to extending the limiting characteristics of *S. caeruleus* so that these fresh isolates can be placed within the species.

Some characteristics of the 24 isolates have been listed (Table V) in the hope that they may assist the taxonomist in the identification of these actinomycetes.

Soil Population

Some characteristics of the soil population are given below.

Association of Acid-sensitivity with Soil Alkalinity

Acid-sensitive actinomycetes were found in 9 of 10 alkaline soils and 1 of 6 acid soils. The single acid soil (No. 6) which yielded an acid-sensitive actinomycete was collected from a region of alkaline soils; hence even this isolate may actually be indigenous to alkaline soils. The frequency of acid-sensitive actinomycetes in alkaline soils is significantly higher (Chi^2 fourfold contingency test for dependency; $P_{1\,d.f.} = .018$) than in acid soils.

Counts

The counts of actinomycetes ranged from approximately 40,000 to 11,000,000 colonies per gram of soil (Table VI). The coefficient of variation for these arithmetic means ranged from 4 to 18%, indicating that the large differences in count can be real. With the exception of soil No. 9, however, there is no correlation between soil pH and the plate count. Soil No. 9 probably represents a special circumstance, since it was a water-saturated soil bordering an alkaline salt lake. The low count observed for this soil suggests that the soil was not suitable for growth of most actinomycetes. James (4) found that a saline soil of Manitoba yielded low counts of bacteria and fungi.

Frequency of Pigmented Actinomycetes

It was noted above that the acid-sensitive actinomycetes were one of two colors, some shade of blue or orange. Most of the actinomycetes which were not unusually sensitive to acidity were either lavender-to-gray or white (Table VI). There was no correlation between pigmentation of the "typical" actinomycetes and the pH of the soil from which they were isolated. This lack of sorting of any of the pigmented typical actinomycetes into one soil type or another is to be expected if the acid and alkaline soils were equally suitable for growth.

Streptomyces of Muskiki Lake

The plating of this soil (soil No. 9, Table VI) produced only one type of actinomycete colony, and the 80 isolates collected from these plates were all sensitive to acidity. If this soil sample were representative of the soil from which it was collected, then it can be concluded that this soil was not suitable for growth or retention of viability of typical actinomycetes. To determine whether these results were reproducible, three additional 1-g lots of the original

20-g sample were plated out. Again all of the colonies on the plates were white and similar in appearance, and the 80 isolates collected from each of the three sets of platings were unusually sensitive to acidity. The counts were also low, the arithmetic mean of 12 plates of each sample being 6100, 5500, and 13,900. The selectivity of this soil was associated with the proximity to salt water since a sample of soil (No. 10) collected 100 yards from the beach yielded a variety of colony types of actinomycetes, only seven of which proved to be acid-sensitive actinomycetes.

The single species remaining viable in, if not growing in, the beach soil must have been tolerant either to the excessive pH or salt concentration (or composition) of the soil. Two representatives of this species and the reference cultures were compared for their ability to grow in 2A—pH 8.5 media supplemented with various quantities of NaCl. Each grew but slightly in 5% NaCl and none grew in 10% or 15% NaCl. No striking difference was noticeable. The salinity of the beach mud is not due solely to NaCl, however, so this laboratory test may not be capable of detecting a greater tolerance to the salts encountered in the natural environment should it exist. An analysis of the water of this lake was made in the fall of 1926 (2). The salts, in parts per million, were $NaHCO_3$ 1451, Na_2CO_3 848, $MgSO_4$ 178,000, Na_2SO_4 113,576, and NaCl 23,500. The specific gravity ranged from 1.25 to 1.27 throughout the year. Even though these determinations were made some years ago, they nevertheless reveal the mixed nature of the salts, and provide a basis for suspecting that if the species in question were highly tolerant of the salt in the soil, this fact might not be detectable by the NaCl test used in the laboratory.

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PREPARATION OF A STABLE NON-INFECTIVE COMPLEMENT-FIXING ANTIGEN FOR HERPES SIMPLEX[1]

JOHN R. POLLEY

Abstract

The soluble antigen of herpes simplex is prepared by grinding the specifically infected chorioallantoic membranes with one volume of saline, storage for 4 days at 4° C to precipitate tissue material, followed by centrifugation for 1 hour at 10,000 r.p.m. (9000 *g*) to improve the specificity. Nine different methods of formaldehyde treatment were tried to determine the best procedure for the destruction of the infectivity while preserving the maximal antigenicity. Methods in which treatment was conducted at pH 6 destroyed considerable antigenicity whereas the methods using a pH of 8.5 were most successful. The method selected to inactivate the virus present in the herpes antigen involves treatment with 0.01% formaldehyde at 37° C for 2 days at pH 8.5, followed by the addition of 0.25 cc of 30% dibasic ammonium phosphate solution per 100 cc of treated antigen. After treatment and neutralization the antigen can be lyophilized for stable storage.

Introduction

For the laboratory diagnosis of a number of virus diseases, such as influenza, mumps, and smallpox, soluble antigens in a stable non-infective form are prepared as described previously (2). The herpes simplex soluble antigen proved to be more difficult to prepare in this manner; prior to the development of the technique being described, it had been used in the form of a live antigen in this Laboratory. For the preparation of influenza vaccines with formaldehyde several methods of treatment have been found suitable, giving a large margin of safety between virus inactivation and loss of antigenicity (3). Therefore, the possibility of preparing a non-infective soluble antigen of herpes simplex by means of one of these methods was investigated.

Materials and Methods

The herpes simplex soluble antigens were prepared by macerating the infected chorioallantoic membranes in one volume of physiological saline. This mixture was centrifuged at low speed and the supernatant fluid was removed and placed at 4° C for 4 days. The mixture was then centrifuged at low speed again and the supernatant fluid was removed as the crude soluble antigen. The optimal speed of centrifugation which can be introduced into the routine preparation of herpes antigens to improve their specificity with minimal loss of potency was investigated. Samples of a common pool of herpes simplex soluble antigen, prepared as described above, were centrifuged at various speeds for 1 hour using the No. 40 rotor in a Spinco model L centrifuge. The supernatant fluids were removed and tested for potency and specificity by complement-fixation. The results are shown in Table I.

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Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada.

The best procedure for the destruction of the infectivity with formaldehyde while preserving the maximal antigenicity was next determined. The nine different methods of formaldehyde treatment found to be successful for the preparation of influenza vaccines were used (3). Since the herpes antigen was known to be relatively labile, these procedures were tried first with the soluble antigens of influenza and mumps to determine if they were applicable. The pH was buffered at the desired values by the addition of monobasic and dibasic sodium phosphate. The final adjustment of pH was made by the addition of 0.1 *N* sodium hydroxide or 0.1 *N* hydrochloric acid. After treatment, the excess formaldehyde was neutralized by the addition of 30% dibasic ammonium phosphate solution (0.25 cc per 10 cc of 0.1% formaldehyde-treated antigen). Samples of each treated antigen were lyophilized, 0.5 cc per 3 cc glass ampoule, using an Edwards centrifugal freeze-dryer. The antigens were reconstituted to their original volume with distilled water and were tested for specific antigenicity by the complement-fixation test and for infectivity by inoculation into the allantoic cavity of 10-day-old chick embryos, as described previously (2).

TABLE I

Effect of centrifugation on the potency and specificity of herpes simplex soluble antigen

Centrifugation	Complement-fixation titer*	Titer of non-specific components
0 (Control)	64	8
5,000 r.p.m. (2,200 *g*)	64	8
10,000 r.p.m. (9,000 *g*)	32	2
15,000 r.p.m. (20,000 *g*)	16	2
20,000 r.p.m. (36,000 *g*)	16	2
30,000 r.p.m. (81,000 *g*)	8	2

*Expressed as the reciprocal of the highest antigen dilution showing 4+ fixation.

It was found that with the influenza and mumps soluble antigens, seven of the methods tried destroyed the infectivity without causing a serious loss of potency. These methods were then used for the treatment of four different herpes antigens. The dried antigens were tested as before. The results of a typical experiment are shown in Table II.

TABLE II

Treatment of herpes simplex soluble antigen with formaldehyde under various conditions

HCHO conc., %	Temp., °C	pH	Time (days)	CF titer	Inf.	CF titer after lyophilization
0 (Control)	—	—	—	32	+	32
0.01*	37	6	7	4	−	2
0.01	37	7	7	16	−	8
0.01	37	8.5	2	32	−	16
0.1	37	6	1	4	−	2
0.1	4	8.5	3	32	−	16
0.1	45	6	1/2	4	−	2
0.25	37	6	1	4	−	2
0.25*	4	8.5	2	16	−	8
0.25	4	7	7	16	−	4

*These procedures did not always produce a non-infective product with influenza and mumps antigens.

Results and Discussion

The routine preparation of the soluble antigens of influenza, mumps, and vaccinia for diagnostic complement-fixation tests in this Laboratory involves centrifugation of the antigen at 20,000 r.p.m. (36,000 *g*) to increase the specificity (4). It can be seen from Table I, however, that this step causes a great loss of potency in the antigen of herpes simplex. It appears that the optimal speed of centrifugation for the elimination of as much of the non-specific reaction as possible before causing a large loss of potency is 10,000 r.p.m. (9000 *g*). Consequently, this step was included in further preparations of herpes simplex soluble antigen.

In Table II are shown the results when nine different methods of formaldehyde treatment were used with portions of a herpes simplex soluble antigen. In general, the herpes antigen proved to be more labile than that of influenza on treatment with formaldehyde, as had been expected. With influenza virus suspensions it was found that the stability of the hemagglutinin to formaldehyde treatment was increased as the pH was decreased to about 6 (3). With the soluble antigen of herpes simplex, however, it can be seen that the complement-fixing component has not been afforded this increased stability by decreasing the pH during treatment. The temperature of the inactivation process is one of the most important factors governing the stability of the antigen. When the formaldehyde concentration was 0.01% and a temperature of 37° C was maintained, the most successful method was the one involving the shortest period of treatment. With a formaldehyde concentration of 0.1%, it was the treatment at the lowest temperature which was the best. At a concentration of 0.25%, again it was the lower temperatures which afforded the most protection to the antigenicity.

The virus of herpes simplex and B virus are similar in some of their physical properties. In this connection it is interesting to compare the above observations on the formaldehyde treatment of herpes simplex soluble antigen with those of Hull and Nash (1) on the formaldehyde treatment of B virus for vaccines. They found that the B virus was also relatively sensitive to heat. Whereas in their case reducing the severity of treatment by lowering the temperature of inactivation did not improve the antigenicity, it was of value with herpes antigen, as shown above. Their solution was to increase the formaldehyde concentration to inactivate the virus before heat degradation occurred. Their selected method of treatment resembles most closely the fourth method in Table II above, which was not successful with herpes antigen.

As a result of the present study it is possible to prepare the soluble antigen of herpes simplex in a stable non-infective form. The antigen is prepared as described above. It is then treated with 0.01% formaldehyde at 37° C at pH 8.5 for 2 days, followed by the addition of 0.25 cc of 30% dibasic ammonium phosphate solution per 100 cc of treated antigen. The antigen can then be lyophilized. Antigens prepared in the above manner have lost no significant potency after storage for 1 year at 4° C.

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OBSERVATIONS SUR LES COULEURS DE LA FLUORESCENCE PRIMAIRE DES MICROORGANISMES[1]

S. SONEA ET J. DE REPENTIGNY

Abstract

Primary fluorescence of microbial cells may show contrasting colors. According to our results, 36 species were divided in two groups: (1) 30 presenting exclusively a yellow-green fluorescence, (2) the 6 others for which reddish cells were observed among yellow-green cells. The species of the second group were: *Candida albicans*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Salmonella typhimurium*, *Sarcina lutea*, and *Serratia marcescens*. In this work, we have deliberately excluded any staining (with fluorochrome or other stain) to avoid possible confusion on the precise meaning of primary fluorescence.

These observations were completed by (*a*) examining, with a "pupillary spectroscope", the light which emerged from the microscope. All the wavelengths of the visible spectrum were, thus, found to be present in the primary fluorescence of the 36 species; (*b*) verifying macroscopically, under the Wood lamp, the resulting dominant color of the fluorescence of washed microorganisms and of their extracts in alcohol, acetone, or water. Thus was confirmed the presence of yellow, green, or reddish fluorescent substances, already found by the previously mentioned methods.

In our experimental conditions (bright condenser, dry objective, and OGI yellow secondary filter), the blue fluorescence was eliminated.

The color of the fluorescence of each species examined could be influenced by culture media, temperature, pH, and age of the cultures, but no reddish fluorescence was observed in the first group. After the smears were prepared, the colors were stable and did not fade for months.

The color variations of the primary fluorescence is suggestive that between microbial cells there are very discreet metabolic differences which could hardly be observed otherwise.

For the classification and the identification of microorganisms, our preliminary findings suggest that the color of primary fluorescence could be a supplementary method, at least for the minority of species producing the reddish fluorescence.

Introduction

Au cours de nos travaux antérieurs sur la détection microscopique (12) et l'intensité (14) de la fluorescence primaire des microorganismes, nous avons toujours observé chez les espèces microbiennes examinées une fluorescence jaune-verdâtre.

D'autre part, des recherches antérieures en histologie ont montré dans les cellules animales et végétales l'existence d'une fluorescence primaire dont les couleurs s'étalent sur toute l'étendue du spectre visible (11). D'ailleurs, beaucoup de substances organiques présentent une fluorescence caractéristique correspondant à leur structure (3, 21) ce qui laisse supposer que les produits métaboliques des microorganismes pourraient eux aussi montrer une fluorescence primaire de couleurs variées ou dont l'intensité est modifiée.

Si l'on considère le nombre considérable des espèces microbiennes et leur capacité de se développer dans des conditions très différentes, on doit s'attendre à trouver parmi les produits qui les constituent une variété de structures capables de produire ou de modifier leur fluorescence primaire.

[1]Manuscrit reçu le 25 mars, 1960.
Contribution du Département de Bactériologie de la Faculté de Médecine, de l'École d'Hygiène, et de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Qué. Cette recherche a été partiellement subventionnée par le Ministère de la Santé de la Province de Québec (subvention fédérale–provinciale à la recherche sur la Santé Publique.)

Il est surprenant que les observations microscopiques de Kaiserling (8), qui a décrit des nuances uniformes de couleurs particulières pour chacune des 14 espèces bactériennes étudiées, n'aient pas donné lieu à des recherches plus poussées sur les couleurs de la fluorescence primaire des microorganismes, durant les quarante années qui ont suivi ses travaux. Une étude restreinte de l'aspect macroscopique des cultures d'*Azotobacter* a déjà montré l'existence d'une fluorescence blanche ou verte selon l'espèce (5, 6).

En poursuivant nos études sur la fluorescence primaire de plusieurs espèces microbiennes, nous avons observé au microscope des cellules à fluorescence rougeâtre parmi d'autres présentant la fluorescence habituelle jaune-verdâtre (15). Ceci nous a encouragés à entreprendre une étude plus détaillée sur la présence des diverses couleurs dans plusieurs frottis d'espèces microbiennes, cultivées dans diverses conditions. D'autre part, nous avons remarqué que pour une même culture nous observions microscopiquement des couleurs diverses et macroscopiquement une seule teinte résultant de l'ensemble des couleurs de tous les microorganismes. Ces observations nous ont poussés à vérifier la répartition et la stabilité des couleurs de la fluorescence primaire dans diverses fractions des cultures liquides de plusieurs microorganismes et à tenter l'extraction des substances qui en sont responsables.

Matériel et Méthodes

(*a*) *Cultures microbiennes.*—36 espèces microbiennes (Tableau I) et 34 souches de *Staphylococcus aureus* ont été cultivées séparément sur membranes de cellophane déposée sur les milieux solides appropriés (gélose au sang, gélose ordinaire, etc. . . .), repiquées ensuite en tubes vissés (150×25 mm) contenant 25 cc d'un des milieux liquides suivants: bouillon ordinaire, "trypticase-soy-broth" (Difco), Muëller-Stone (18), Hornibrook et un milieu semi-synthétique à base d'hydrolysat de caséine mis au point dans nos laboratoires et choisi à cause de sa faible fluorescence. Nous avons généralement employé des cultures pures de 17 heures, à 37° C, pour la majorité des espèces, et des cultures pures de 72 heures à 22° C, pour les espèces qui poussent mieux à la température de la pièce. Nous avons aussi étudié des cultures en phase de croissance exponentielle, âgées de 6 heures, et aussi de vieilles cultures de 10 jours.

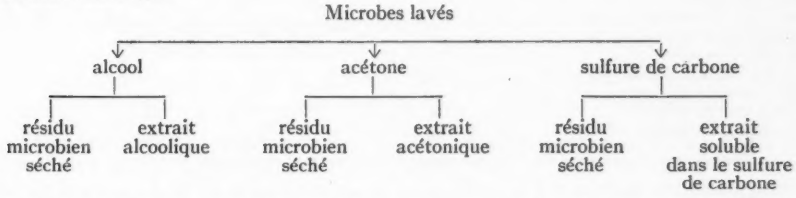
(*b*) *Préparation des frottis.*—La méthode de préparation des frottis a déjà été décrite dans une note précédente (14).

(*c*) *Observation visuelle au microscope.*—Les frottis ont été examinés selon notre méthode de microscopie à fluorescence (12, 14) qui est caractérisée surtout par l'emploi du condensateur à fond clair et d'un objectif sec, lequel, dans le présent travail, a été le 42×, muni d'un filtre Euphos. A cause de l'emploi des filtres jaunes OGI 2.5 mm et Euphos 0.17 mm, qui éliminent les rayons de longueur d'onde plus courte que 500 mμ, la fluorescence primaire bleue, présente chez toutes les espèces examinées, a été exclue de notre expérimentation. Ces observations ont été exécutées dans une obscurité relative.

(*d*) *Spectroscopie au microscope.*—Les mêmes frottis qui ont été examinés à l'œil ont été soumis à l'analyse spectrale en remplaçant l'oculaire par le microspectroscope Zeiss "pupil spectroscope" mais en conservant le même système de filtres qui laissent passer la lumière visible de la fluorescence primaire à partir de la longueur d'onde de 500 mμ jusqu'au rouge. Dans ces conditions, nous avons enregistré deux données en longueurs d'onde, soit: l'étalement du spectre de la fluorescence primaire et la fraction dominante, cette dernière par extinction.

(*e*) *Contrôle macroscopique de la fluorescence primaire.*—Les frottis et les tubes contenant les cultures totales et les fractions qui en dérivent (voir plus bas), ont été examinés à l'obscurité sous la lampe de Wood, en intercalant, comme dans le cas des observations au microscope, un filtre jaune entre l'œil et les spécimens irradiés.

(*f*) *Fractionnement des cultures.*—En vue d'étudier la répartition de la fluorescence primaire dans diverses parties des cultures microbiennes, nous avons fait appel à la centrifugation et à deux lavages des microorganismes par l'eau distillée. Par ces méthodes nous avons obtenu séparément: le liquide surnageant, le culot des bactéries non lavées, une première et une deuxième série d'eaux de lavage, et finalement des bactéries lavées. Ces dernières ont été extraites par des solvants organiques pendant 48 heures, à 20° C selon le schéma suivant:



A partir des mêmes microbes lavés, nous avons préparé les parois cellulaires selon la méthode de Salton et Horne (16).

Résultats

Dans nos conditions expérimentales, la fluorescence primaire des cellules microbiennes provenant de cultures pures présente des différences de couleurs dans un même champ microscopique. Le plus souvent, il s'agit de légères variations à partir du jaune au vert, couleurs les plus fréquentes de la fluorescence primaire et constamment trouvées chez les microorganismes étudiés. Pour certaines espèces, parmi des éléments cellulaires verdâtres, on trouve aussi d'autres éléments d'une couleur rougeâtre: orangée, rosée ou rouge. Cependant cette couleur ne peut être constatée macroscopiquement dans les cultures totales, qui ont toujours présenté une fluorescence dominante verte dans nos conditions expérimentales; pour la voir, il faut examiner des microorganismes isolés ou des masses bactériennes lavées. Nos résultats sont présentés en deux groupes d'espèces, lesquelles sont comparées en fonction de la présence ou de l'absence de cellules rougeâtres lorsque cultivées dans des conditions similaires (même âge de culture, mêmes milieux, etc.).

TABLEAU I

Espèces microbiennes étudiées

Groupe I Corps microbiens à fluorescence exclusive jaune-verdâtre*	Groupe II Corps microbiens à fluorescence rougeâtre et jaune-verdâtre*
Aerobacter aerogenes	*Candida albicans*
Bacillus megaterium	*Pseudomonas aeruginosa*
Bacillus subtilis	*Saccharomyces cerevisiae*
Bacterium anitratum (B5W)	*Salmonella typhimurium*
Corynebacterium diphtheriae	*Sarcina lutea*
Corynebacterium pseudodiphtheriticum	*Serratia marcescens*
E. coli 055:B5	
E. coli 0111:B4	
E. coli	
Gaffkya tetragena	
Klebsiella pneumoniae	
Listeria monocytogenes	
Mycobacterium phlei	
Mycobacterium ranae	
Mycobacterium smegmatis	
Paracolobactrum aerogenoides	
Proteus mirabilis	
Proteus morganii	
Proteus vulgaris	
Salmonella gallinarum	
Salmonella hirschfeldii	
Salmonella newport	
Shigella boydii	
Shigella dysenteriae	
Shigella flexneri	
Shigella sonnei	
Staphylococcus aureus	
Streptococcus faecalis	
Streptococcus pyogenes	
Streptococcus salivarius	

*Les filtres jaunes employés ont éliminé la fluorescence bleue, présente chez toutes ces espèces.

TABLEAU II

Les couleurs de la fluorescence primaire des microbes lavés et de leurs extraits (résumé des résultats obtenus*)

	Groupe I	Groupe II
Observation visuelle au microscope	Cellules à fluorescence exclusivement jaune-verdâtre	Cellules rouges parmi des cellules jaunes-verdâtres
Microspectroscopie	Toutes les longueurs d'onde du spectre visible	Toutes les longueurs d'onde du spectre visible
Observation macroscopique des frottis sous la lampe de Wood	Jaune-verdâtre	Rougeâtre ou jaune-verdâtre selon la proportion des éléments rougeâtres
Observation macroscopique des extraits alcoolique et acétonique, sous la lampe de Wood†	Jaune-verdâtre	Rougeâtres (passant lentement au vert)

*Ce tableau résume les observations visuelles confirmées d'ailleurs par des photographies en couleur.
†Incolores en lumière ordinaire.

(*a*) *Microorganismes présentant exclusivement une fluorescence primaire jaune-verdâtre*

Trente des trente-six espèces examinées (Tableau I) ont toujours montré une fluorescence jaune-verdâtre microscopiquement et macroscopiquement, indépendamment des variations dans les conditions de cultures et d'examens.

On retrouve cette même couleur dans leurs cultures liquides totales, leurs surnageants, et dans leurs extraits à l'eau, à l'acétone et à l'alcool (Tableau II).

Les parois cellulaires et les matières cytoplasmiques de quelques espèces typiques de ce groupe, dont le staphylocoque, ont présenté la même fluorescence jaune-verdâtre, mais plus pâle. Dans aucune de nos conditions expérimentales, même dans de très vieilles cultures, nous n'avons pu observer dans ce groupe d'espèces, de cellule à fluorescence rougeâtre.

La fluorescence macroscopique des masses bactériennes de *S. sonnei* et de *C. pseudodiphtériticum* présentait une teinte jaune-verdâtre dans quatre des cinq milieux employés, mais, d'une façon exceptionnelle, la fluorescence a été jaune-orange lorsque les bactéries ont été cultivées respectivement dans le milieu semisynthétique ou dans le bouillon ordinaire.

Pour vérifier la stabilité de la couleur de la fluorescence des souches à l'intérieur d'une espèce, nous avons examiné 34 souches de *Staphylococcus aureus* et nous avons retrouvé constamment la même teinte jaune-verdâtre.

(*b*) *Microorganismes présentant une fluorescence primaire rougeâtre*

Six des 36 espèces examinées (voir tableau I) ont montré des cellules à fluorescence rougeâtre (orange, rosée ou rouge), en proportion variable parmi des éléments jaune-verdâtres. Seules les masses microbiennes de ce groupe ont montré macroscopiquement, en tubes ou en frottis, dans la plupart des cas, une fluorescence d'une teinte uniforme rougeâtre. Exceptionnellement selon le milieu ayant servi à la culture, il arrivait que la couleur dominante fût jaune-verdâtre.

Les variations dans la proportion des couleurs ont été fonction des espèces examinées et de certaines conditions de culture. Parmi les milieux employés, celui de Muëller, modifié par Stone, a produit le moins d'éléments rougeâtres. Les autres milieux en ont produit en proportion variable.

L'âge de la culture semble avoir une influence sur la proportion des éléments rougeâtres. Nous avons constaté une plus grande proportion de cellules rougeâtres dans les cultures de 10 jours de *S. lutea* par rapport aux cultures de 48 heures et plus jeunes pour arriver à une proportion négligeable dans les cultures de 6 heures.

La congélation modifie la couleur de la fluorescence primaire de deux des espèces examinées. Tous les éléments microbiens de nos souches de *Sarcina lutea* et de *Pseudomonas aeruginosa*, exposés à l'air en couche mince, à $-5°$ C, acquièrent une fluorescence rougeâtre intense, qui se transforme en vert assez vite sous l'action combinée de la température de la pièce et des rayons bleus ou ultra-violets de notre installation microscopique. Ces transformations de couleurs chez ces deux espèces sont réversibles en fonction de la température. Nous n'avons pu obtenir des résultats semblables avec plusieurs autres espèces.

Un pH qui s'éloigne de la neutralité semble diminuer d'une façon constante la proportion des éléments à fluorescence rougeâtre.

Dans les extraits alcooliques, acétoniques ou aqueux des corps bactériens lavés de *P. aeruginosa*, *S. typhimurium* et *S. lutea*, il a toujours été possible de retrouver des substances à fluorescence rougeâtre (Tableau II). La couleur rouge de ces extraits peut changer au vert avec le temps, beaucoup plus facilement pour les extraits aqueux, que pour les extraits alcooliques ou acétoniques; cependant, la couleur rougeâtre de ces derniers peut changer au vert sous l'action du sulfure de carbone.

Sous l'action de l'alcool et de l'acétone, les culots microbiens ne changeaient pas de couleur, ce qui montre que l'extraction n'était que partielle et que ces solvants ne modifiaient pas la couleur de leur fluorescence.

(*c*) *Résultats communs aux deux groupes de microorganismes*

Contrairement aux microorganismes lavés, les cultures liquides totales ainsi que leurs surnageants après centrifugation, ont présenté, sans exception, une fluorescence macroscopique jaune-verdâtre difficile à différencier de celle des milieux de culture non ensemencés; cette couleur jaune-verdâtre est prépondérante parce que nous éliminons le bleu dans nos conditions expérimentales.

La couleur de la fluorescence des frottis des microorganismes ne subit pas de modifications lorsque ceux-ci sont conservés à l'obscurité et à la température de la pièce pendant cinq mois.

Le sulfure de carbone n'extrait pas de substances fluorescentes décelables dans nos conditions expérimentales.

L'observation au microscope du spectre de la fluorescence primaire des diverses espèces microbiennes et de leurs fractions a montré la présence constante de longueurs d'onde allant sans interruption du vert au rouge, même dans le cas où le frottis ne contenait pas d'éléments rougeâtres. L'étalement du spectre dans le rouge montrait une limite supérieure qui variait en fonction des espèces, des souches et des diverses conditions de culture entre 650 et 700 mμ; ce résultat a été obtenu également avec, à la source, un filtre (UGI, 2 mm) et avec notre système habituel de deux filtres. Les limites supérieures des longueurs d'onde, dominant en intensité, obtenues par extinction (occulaire Ramsden à disphragme), ont varié entre 570 et 610 mμ, dans le cas d'un filtre à la source, et entre 570 et 590 mμ, avec les deux filtres. Cette même limite supérieure, dans le cas des surnageants des cultures, a varié entre 640 et 690 mμ, pour toutes les espèces cultivées sur cinq milieux différents.

Discussion

On sait qu'il existe une relation étroite entre la structure chimique des substances organiques et les propriétés de leur fluorescence (3,4). Cette relation a été vérifiée dans le cas de plusieurs protéines et de leurs constituants (21). Les couleurs différentes observées dans la fluorescence primaire des microorganismes sont très probablement dues à des substances de structures chimiques différentes. Cependant, nous n'avons pas essayé de les analyser. La fluorescence rougeâtre semble provenir uniquement des cellules bactériennes, puisqu'elle n'est pas décelable dans nos milieux de cultures liquides stériles et

elle ne devient macroscopiquement visible qu'après l'élimination du surnageant des cultures.

Il est surprenant que des bactéries provenant d'une même culture pure puissent présenter, sur le même champ microscopique, les unes une fluorescence rougeâtre, les autres, verdâtre. Pennington (10) a montré à l'aide d'une autre technique, une hétérogénéité des cellules bactériennes, même dans de jeunes cultures.

La présence et la proportion des cellules rougeâtres dans les espèces du deuxième groupe, et leur couleur résultante observée à l'examen microscopique varient selon les modifications de métabolisme qui sont causées par des conditions de cultures différentes (milieux de culture de composition différente, âge, température, etc.). Ceci laisse entrevoir la possibilité que l'observation de la fluorescence primaire pourrait constituer une méthode supplémentaire d'étude, pour détecter des variations du métabolisme microbien dans les microorganismes intacts ou dans des fractions.

L'observation de Barnard et Welsh en 1936 (1) d'après laquelle des parois cellulaires ne seraient pas fluorescentes, diffère de nos résultats. Elle s'explique probablement par le fait qu'ils ont fait cette étude à une époque où les parois cellulaires n'avaient pas encore été isolées et examinées comme telles. Nos observations ont été favorisées par l'existence d'appareils récents améliorés, surtout de sources puissantes et stables de rayons ultra-violets et il est probable que les installations plus perfectionnées qui seront disponibles dans l'avenir permettront de préciser davantage les propriétés de la fluorescence primaire.

Pour ne pas donner lieu à des équivoques, dans ce travail sur la fluorescence primaire, nous avons exclu systématiquement toute coloration soit avec des substances fluorescentes soit avec des colorants ordinaires. D'ailleurs les résultats éventuels obtenus par de telles colorations ne permettraient tout au plus qu'à établir des corrélations incertaines avec le phénomène si peu connu de la fluorescence naturelle des microorganismes.

Le microspectroscope a montré, dans la fluorescence primaire de tous les microorganismes examinés, la présence de toutes les longueurs d'onde correspondant probablement à une très importante gamme de produits synthétisés. Il est clair que le microspectroscope tel que nous l'avons employé donne peu d'informations pour différencier les couleurs des microorganismes. L'examen de la fluorescence à l'œil nous a permis de mieux distinguer les couleurs d'une espèce à l'autre. On peut pourtant concevoir que par un choix de longueurs d'onde de la source lumineuse, et par l'utilisation d'un microspectroscope adapté spécialement pour des lectures prises entre 530 et 730 $m\mu$, l'examen au microspectroscope pourrait constituer une méthode objective, complémentaire ou supérieure à l'examen microscopique à l'œil.

Nos résultats au microspectroscope ont pu être influencés par les variations de l'intensité de la fluorescence des spécimens. Il est probable que des différences plus significatives entre les espèces auraient été obtenues au microspectroscope si les champs microscopiques avaient été choisis non pas en fonction de volumes égaux mais en fonction de l'intensité de leur fluorescence.

L'examen macroscopique, sous la lampe de Wood, nous a donné moins d'informations que l'examen microscopique sur les détails des couleurs de la fluorescence primaire. D'une part, la participation de la fluorescence verdâtre des milieux de culture eux-mêmes et de certains pigments fluorescents diffusibles déjà connus (7, 9, 17) qu'ils renferment est tellement prépondérante dans l'aspect de la culture totale examinée sous la lumière de Wood qu'il faut pratiquement l'éliminer par centrifugation pour observer les vraies couleurs des bactéries après leur séparation. La couleur de la fluorescence des bactéries lavées, si elle est différente, peut dominer celle des traces de surnageants de culture qui peuvent y subsister. D'autre part, la teinte unique finale d'une masse bactérienne est la résultante des couleurs de chacune des cellules. Quand la teinte résultante est rougeâtre, elle correspond nécessairement à une proportion assez élevée de cellules rougeâtres. L'examen macroscopique des masses bactériennes lavées, en tube ou en frottis épais, a constitué pour nous un moyen rapide de confirmer nos observations microscopiques d'éléments rougeâtres et pour dépister de nouvelles espèces capables d'en posséder. Nous pensons que cette méthode ajoutée à l'examen microscopique en fluorescence pourrait s'ajouter parfois, même si c'était pour un nombre limité d'espèces, aux critères actuels morphologiques, biochimiques et sérologiques pour la classification et l'identification de certains microorganismes.

Le lavage des microorganismes par l'eau distillée enlève pratiquement les dernières traces fluorescentes du milieu de culture et aussi mais partiellement certaines substances fluorescentes d'origine bactérienne. Dans le cas où les microorganismes contiennent une proportion suffisante d'éléments rougeâtres, cette dernière couleur peut être retrouvée dans les eaux de lavage, et dans les extraits alcooliques et acétoniques. Cependant, nous n'avons pas cherché à faire des études complètes sur l'extraction des substances fluorescentes présentes dans les cultures des microorganismes. Ces substances fluorescentes ainsi extraites semblent pouvoir s'altérer beaucoup plus facilement qu'à l'intérieur des bactéries car la couleur des extraits peut changer à la température de la pièce assez facilement. Au contraire, nous avons vu que les bactéries gardent leur teinte, à l'exception de quelques espèces soumises à l'action du froid, et qui font présentement l'objet d'une étude à part. Il est possible qu'à l'intérieur des bactéries ces substances soient plus complexes.

Le pigment à fluorescence verte du groupe Pseudomonas–Fluorescens (19, 20) semble différent des substances à fluorescence rougeâtre que nous avons trouvé chez plusieurs bactéries dont *Pseudomonas aeruginosa*. D'une part, le premier est de fluorescence verte dans toutes les conditions expérimentales, insoluble dans les solvants organiques (9) et diffusible dans le milieu de culture (surnageant pour les cultures liquides). D'autre part, les substances responsables de la fluorescence rouge qui peut passer au vert en certains cas, sont solubles dans plusieurs solvants organiques et se retrouvent surtout au niveau des microorganismes quand on a retiré le milieu de culture. D'autres auteurs ont étudié la fluorescence des porphyrines, des bactériochlorophyles et des flavines chez les bactéries (4). Si on refère à ces travaux, la fluorescence

jaune-verdâtre que nous avons observée, pourrait être rapprochée plutôt de celle des flavines. Par contre, la fluorescence rougeâtre nous fait penser plutôt à celle des bactériochlorophyles et des porphyrines.

Même si on est habitué à associer les bactériochlorophyles avec les bactéries photosynthétiques, dans le sous-ordre des Rhodobacteriineae, très éloignées des espèces étudiées par nous, il n'est pas impossible que, dans certaines conditions, des structures semblables puissent se trouver chez d'autres microorganismes.

D'une façon générale, il semble y avoir une répartition des couleurs de la fluorescence primaire analogue à la répartition de certains antigènes bactériens dans des cultures liquides déjà étudiées (2, 13). Toutefois, il est probable que les substances fluorescentes extraites par les solvants organiques non dilués ne sont pas des antigènes.

Le problème des couleurs de la fluorescence primaire des microorganismes est très complexe et sujet à plusieurs limitations. L'œil humain étant particulièrement sensible au jaune, l'observation visuelle peut passer à côté d'un rouge faible au milieu d'éléments jaunâtres. Dans le même ordre d'idée, il faut interpréter avec prudence les photographies en couleurs, car leurs teintes peuvent varier avec la sensibilité sélective du type de film utilisé, avec l'intensité de la fluorescence et le temps d'exposition.

Il est possible que d'autres milieux que ceux que nous avons utilisés favoriseraient davantage la différenciation à l'aide des couleurs de la fluorescence. De tels milieux existent déjà pour la mise en évidence sous la lumière ordinaire des pigments visibles, lesquels sont très différents des substances fluorescentes responsables de la fluorescence primaire.

Conclusions

1. Notre étude démontre que les 36 espèces microbiennes examinées présentent au microspectroscope une fluorescence primaire s'étalant sans interruption sur toutes les longueurs d'onde du spectre visible.

2. L'examen visuel au microscope nous permet de voir des cellules microbiennes de couleurs différentes, même en cultures pures. Par contre, l'examen macroscopique d'un grand nombre de microorganismes montre une fluorescence à teinte unique, dominante, résultant de ces couleurs diverses.

3. Dans nos conditions expérimentales, la fluorescence primaire jaune-verdâtre se retrouve chez toutes les espèces microbiennes étudiées et pour la majorité de celles-ci, cette couleur est exclusive. Pour ce dernier groupe, la couleur de la fluorescence primaire n'est évidemment pas un critère sur lequel on peut se baser pour distinguer diverses espèces. La fluorescence bleue est aussi très répandue, mais elle ne pouvait pas être observée à cause du système de filtres employé.

4. Chez quelques espèces microbiennes, nous avons observé des cellules à fluorescence rougeâtre, propriété plus facile à mettre en évidence chez les microorganismes lavés. Pour ce groupe d'espèces, l'examen au microscope et l'examen des masses bactériennes sous la lampe de Wood pourraient constituer des critères pour leur classification et leur identification.

5. Les couleurs diverses de la fluorescence primaire microbienne correspondant à des structures chimiques différentes, peuvent être retrouvées dans des extraits aqueux, alcooliques et acétoniques, de microorganismes lavés.

6. En général, les couleurs de la fluorescence des corps microbiens sont stables. Les extraits fluorescents peuvent changer de couleur avec le temps.

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THE EFFECT OF HORMONES ON HELA CELLS INOCULATED WITH INFECTIOUS HEPATITIS SPECIMENS[1]

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Abstract

HeLa cells have been studied in media supplemented with prednisolone, hydrocortisone, testosterone, oestradiol, progesterone, and aldosterone, singly and in combination. Twelve acute phase infectious hepatitis specimens did not cause a consistent degenerative effect in hormone-enriched cultures. Repeated inoculation with two virus specimens to cultures with testosterone also failed to produce a cytopathogenic effect.

The effect of hormones in tissue culture has received considerable study. In 1938 Solders (24) investigated the stimulatory effect of hypophyseal extracts on heart fibroblasts. Cortisone, hydrocortisone, 17-hydroxycortisone, and desoxycorticosterone have been studied by a number of workers (1–3, 10, 11, 13, 17, 22). Other investigations have included parathyroid gland extracts (9), insulin (6, 23), oestrone, and compound "F" (5, 14, 16). However, it has not been until recently that virus propagation has been studied in hormone-enriched cultures. Kilbourne and Tateno (18) have demonstrated that when chorioallantoic membranes of chick embryos were cultured in a medium supplemented with cortisone, significantly higher titers of influenza B virus were obtained. They suggested that the increase in virus yield induced by cortisone may have resulted from prolonged survival of membrane cells permitting continued viral multiplication. Glasgow *et al.* (12) prepared tissue cultures of spleen from guinea pigs inoculated with mumps, and noted that cortisone protected the cells from the cytotoxic action of the virus. Likar and Wilson (20) have added insulin to culture media with monkey kidney, HeLa cells, and amnion cells, and determined that there was an increased production of type 1 poliovirus. Fisher and Fisher (7) observed that a herpes virus – cortisone association in HeLa cells altered carbohydrate metabolism resulting in a more marked acid production and significant increases in virus titers. The striking neonatal susceptibility of humans and suckling mice to Coxsackie virus infections with frequent cardiac complications has been suggested as an effect of higher circulating hormonal levels (4, 19, 21). Franklin and Sinclair (8) have added prednisolone to cultures of human adult tissues and monkey tissues, observing that growth of liver specimens was restricted to epithelial-like cells and that a slight growth stimulus resulted. However, the addition of prednisolone to culture media did not predispose cells to degenerative changes in the presence of acute phase infectious hepatitis specimens.

Although tissue culture experiments with acute phase infectious hepatitis specimens have been unsuccessful, it was considered that modifications of

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existing techniques should be studied further. Therefore, experiments were carried out in HeLa cells, using varying concentrations of six hormones, both singly and in certain combinations.

Technical Methods

HeLa cultures.—Cells were grown in nutrient media supplemented with 10% inactivated sheep serum. The growth media have included synthetic mixtures 217 (8), 1066 (15), and Hanks-Eagle's mixture. Stock cultures in 8-oz. milk dilution bottles were dispersed with 0.1% trypsin, and subsequently added to other stock bottles and roller tubes. Confluent stock cultures and inoculated roller tubes were maintained in mixture 1066 supplemented with 10% tryptose–phosphate broth.

Hormones.—Hormones selected for this study included prednisolone, hydrocortisone acetate, testosterone propionate (alcoholic and aqueous preparations). Pharmaceutical preparations were used in each case, with the exception of aldosterone, which was kindly prepared by Dr. A. Gornall, Department of Pathological Chemistry, University of Toronto.

Inocula.—The inocula were obtained through the courtesy of Dr. J. C. Sinclair of this department, and consisted of (*a*) presumed normal serum and 10% faecal extract, and (*b*) acute phase infectious hepatitis (IH) serum and 10% faecal extract, which were obtained from patients within 7 days of onset of clinical symptoms. Sera were inoculated as 0.2 ml of 1:4 saline dilutions and faecal extracts as 0.1 ml of 1:5 saline dilutions per culture.

Preparation of cultures.—Roller tube cultures of HeLa cells were prepared in groups of 10 with nutrient medium and 10% sheep serum supplemented with the hormone preparation in which the final concentration was 10, 1.0, 0.1, and 0.01 mg%. Aldosterone was added in final concentrations of 0.1, 0.01, and 0.001 mg%. Additional experiments with aqueous testosterone propionate were carried out in which the final hormone concentrations were 0.75, 0.50, and 0.25 mg%. Two experiments in which 0.1 mg % testosterone propionate was included in the medium were selected for repeated inoculation. To the cultures was added 0.1 ml of diluted faecal extract or 0.2 ml of undiluted (IH) serum at each passage in an attempt to increase the concentration of virus.

The influence of single hormones.—Each of the hormones listed above was first studied for evidence of toxicity. If a cell sheet became confluent, the cultures were divided into two equal groups and inoculated with normal and acute phase (IH) specimens. All cultures were deep frozen at the termination of the experiment, then thawed and harvested. Multiple passage inocula were subsequently added to cultures which were prepared under similar conditions.

Results

(1) A concentration of 10 mg% prednisolone appeared to be slightly toxic to HeLa cells, whereas lower concentrations resulted in a longer period of survival. Cells in prednisolone were also larger, had a rather translucent appearance, and cell peripheries were better defined than those in which the hormone was omitted.

(2) There were no apparent differences between the use of hydrocortisone acetate and prednisolone.

(3) In 10 and 1.0 mg% concentrations, testosterone propionate destroyed HeLa cultures; 0.1 and 0.01 mg% concentrations caused the formation of a few more round cells than control cultures. Control cultures with an equal amount of alcohol were better than those without alcohol. There was no apparent difference between the use of alcoholic and aqueous testosterone propionate.

(4) Oestradiol monobenzoate affected the cells in a manner similar to that of testosterone propionate.

(5) Media containing a final concentration of 10 mg% progesterone caused a loss of confluency and considerable granularity; 1.0 mg% progesterone caused some granularity, whereas only a slight effect was evident in the presence of 0.1 and 0.01 mg% progesterone.

(6) Aldosterone (0.1 mg%) caused some cellular granularity and slight degeneration at the edge of the sheet. Lower concentrations were without an apparent effect.

(7) As many as six and not fewer than two passages of acute (IH) specimens were carried out in the presence of each hormone concentration that did not cause a loss of confluency. Two acute phase faecal extracts and nine acute phase (IH) sera were used, as well as comparable controls. There were no consistent degenerative effects seen in the presence of any of these hormones. However, well-defined cytopathogenic effects were seen occasionally in some passages when 0.1, 0.25, 0.50, and 0.75 mg% testosterone propionate or 0.1 mg% progesterone was added to the media. The effects in progesterone were not as numerous as those in testosterone cultures.

(8) Repeated inoculation of an acute faecal extract and an acute serum at each passage level in two experiments failed to enhance degenerative effects during five passages, when 0.1 mg% testosterone propionate was added to the medium.

The Influence of Combinations of Hormones

Since hormones used singly did not potentiate any consistent viral pattern in HeLa cells, it was considered that combinations of hormones might predispose the cells to degenerative processes when inoculated with acute (IH) specimens. Experiments with combinations of hormones were set up as in the following examples: (1) growth medium (GM); (2) GM and 1 mg% prednisolone (P); (3) GM and 1 mg% testosterone propionate (TP); (4) GM and 0.1 mg% TP; (5) GM, 1.0 mg% P and 1.0 mg% TP; and (6) GM, 1.0 mg% P and 0.1 mg% TP. Other combinations at the same concentrations included (*a*) prednisolone and oestradiol monobenzoate, (*b*) prednisolone and aldosterone (at 0.1 and 0.01 mg%), (*c*) prednisolone and progesterone, and (*d*) testosterone propionate and progesterone. The cultures were inoculated as described above.

Results

(1) When 1 mg% prednisolone was used in combination with 1 mg% testosterone propionate or oestradiol monobenzoate, prednisolone appeared to protect the cells against the toxic effects produced by these hormones in HeLa cells.

(2) When 1 mg% prednisolone was used in combination with 0.1 mg% of these hormones, HeLa cells resembled those in prednisolone alone.

(3) A 1 mg% concentration of prednisolone did not protect HeLa cells against the granular effects caused by 1 mg% progesterone; rather the combination of these hormones resulted in more granular cells than in single hormone cultures.

(4) When 0.1 mg% progesterone was combined with 0.1 mg% testosterone propionate, the effects were similar to those for single hormone cultures.

(5) As many as five passages and not less than two passages were carried out with two acute phase faecal extracts and four acute phase (IH) sera with these hormone combinations, with comparable controls. There were no consistent degenerative effects seen under any of these conditions.

Discussion

Although the appearance of degenerative effects in HeLa cell cultures inoculated with acute phase infectious hepatitis specimens was inconsistent, a clearly defined cytopathogenic effect was occasionally observed. This was evident in some passages when 0.1, 0.25, 0.50, and 0.75 mg% testosterone propionate or 0.1 mg% progesterone was added to the medium but bore no relation to a particular concentration of hormone. Degenerative changes did not occur in cultures containing prednisolone, hydrocortisone, oestradiol, or aldosterone.

Various combinations of the above hormones did not produce a viral effect. Furthermore, repeated inoculation of fresh specimen aliquots along with harvested passage material has failed so far to enhance degenerative changes in cultures with testosterone propionate.

Conclusions

(1) HeLa cultures have been studied in the presence of six hormones in final concentrations of 0.01 to 10 mg% in growth media. Hormones used in this series included prednisolone, testosterone propionate, hydrocortisone acetate, oestradiol monobenzoate, progesterone, and aldosterone. Combinations of these hormones have also been studied in a similar manner.

(2) When cultures supplemented with hormones were inoculated with acute phase infectious hepatitis specimens, consistent degenerative effects were not observed. Hormones were used singly and in combinations. Two acute phase faecal extracts and twelve acute phase sera were passed serially under these conditions for not less than two and for as many as six passages. Two specimens which were inoculated at each of five passages failed to enhance degenerative effects which were seen in some cultures containing testosterone propionate.

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TURBIDITY OF SUSPENSIONS AND MORPHOLOGY OF RED HALOPHILIC BACTERIA AS INFLUENCED BY SODIUM CHLORIDE CONCENTRATION[1]

DINAH ABRAM[2] AND N. E. GIBBONS

Abstract

The optical densities of suspensions of cells of *Halobacterium cutirubrum*, *H. halobium*, or *H. salinarium*, grown in media containing 4.5 *M* sodium chloride, increase as the salt concentration of the suspending medium decreases, until a maximum is reached at about 2 *M*; below this concentration there is an abrupt decrease in optical density. The cells are rod shaped in 4.5 *M* salt and change, as the salt concentration decreases, through irregular transition forms to spheres; equal numbers of transition forms and spheres are present at the point of maximum turbidity, while spheres predominate at lower salt concentrations. Cells suspended in 3.0 *M* salt, although slightly swollen, are viable, but viability decreases rapidly with the more drastic changes in morphology at lower salt concentrations. Cells grown in the presence of iron are more resistant to morphological changes but follow the same sequence. Cells "fixed" with formaldehyde, at any point in the sequence, act as osmometers and do not rupture in distilled water although their volume increases 10–14 times. The results indicate that the red halophilic rods require a high sodium chloride content in their growth or suspending medium to maintain a rigid cell wall structure.

Introduction

It has been known for some time that the red halophilic bacteria are very susceptible to osmotic changes. In water, dilute sodium chloride solutions and solutions of dextrose, sucrose, and salts other than sodium chloride, the cells swell and lyse resulting in a clearing of the suspensions. However, unexpected variations and changes in the turbidity of suspensions of extreme halophiles in decreasing concentrations of salt have been noted which could not be interpreted in osmotic terms alone.

In this paper data are presented to show the effect of salt[3] concentration in the growth and suspending media on the turbidity of suspensions of some red halophilic rods, and to relate these changes to the morphology of the cells. It was thought that more information regarding the osmotic sensitivity of these organisms and of the protective effect of salt may lead to a better understanding of bacterial halophilism.

Methods

Halobacterium cutirubrum, a red-pigmented rod growing in salt concentrations ranging from 5 to 3 *M*, was used as a typical extreme halophile. *H. salinarium* and *H. halobium* were used occasionally for comparison but as the results were similar only those obtained with *H. cutirubrum* are reported.

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[3]Salt throughout this paper refers to sodium chloride.

The growth medium contained casamino acids (Difco) 5 g, proteose–peptone (Difco) 5 g, yeast extract (Difco) 10 g, magnesium sulphate heptahydrate 20 g, potassium chloride 2 g, trisodium citrate dihydrate 3 g, sodium chloride as indicated, distilled water to 1000 ml, and was adjusted to a final pH of 7.2–7.4. Iron, when used, was added after sterilization as ferrous sulphate to give a final concentration of 10 p.p.m. (9).

Cultures were incubated on a rotary shaking machine at 37° C and the cells harvested after 40–43 hours by centrifuging in a refrigerated centrifuge at 6000–8000 r.p.m. (4600–8000 × *g*). The cells were washed once and resuspended in a solution of the same sodium chloride concentration as the growth medium. The concentration of this stock suspension was adjusted so that when diluted 1:100 or 1:200 with a 4.5 *M* salt solution the optical density was about 0.1–0.25. The stock suspensions were stored at 5° but used no longer than 3 days.

The experimental procedure consisted of adding one or two drops of the stock suspensions to 10-ml aliquots of salt solutions of various concentrations, mixing thoroughly, and measuring the turbidity at intervals during incubation usually at room temperature (ca. 25° C). Turbidity measurements were made at 660 mμ against a blank of the same suspension in distilled water unless otherwise noted. Cells lyse and disappear in water giving a clear solution. Not more than 10 tubes were prepared at one time, which allowed the first reading to be taken 2 minutes after the addition of the cells. Turbidity values are presented as the optical density, or as a percentage of the optical density of a suspension in a sodium chloride solution of the same concentration as in the growth medium.

"Fixed" cells were prepared by diluting the stock solution in salt solutions to an O.D. of 0.3–0.5, adding sufficient 37% formaldehyde saturated with salt to give a final concentration of approximately 2% formaldehyde, and allowing the suspension to stand overnight at room temperature. The cells were then washed twice and resuspended as a thick suspension in salt solution of the same concentration as the original. This stock suspension could be kept up to 4 weeks at 5°. As these cells do not lyse in water, turbidity measurements were made against a water blank.

Microscopic examinations were made on wet preparations with a Spencer phase contrast microscope. Cells were immobilized for photographing by adding 2% carboxymethylcellulose, type 70 high (Hercules Powder Co.) to the salt solutions.

Viable cells were counted by the drop plate method using pipettes calibrated to deliver 50 drops of water per milliliter. No correction was made for the density of the salt solutions. All dilutions except the last were made in broth containing the same concentration of salt as the growth medium; the final dilution was made in broth with salt at the concentration under test and cells were allowed to remain in it for 1 hour at room temperature before plating. Counts were made after incubation at 37° for 7 days on agar containing 4.5 *M* salt. Total cell counts were made in a Petroff-Hauser counting chamber.

Results

Cell Morphology

Cells grown in 4.5 *M* salt and suspended in 4.5 *M* salt solutions are uniform and rod shaped (Fig. 1). Cells grown in 3.0 *M* salt are irregular in size and many swollen forms are present (Fig. 2). Cells of these organisms are very susceptible to mechanical damage but cells grown in 3.0 *M* salt are more susceptible than those grown in 4.5 *M* salt. The rods can be changed to spheres by pressing on the cover slip of a wet mount. Centrifugation also causes damage and after two or three centrifugations at high speed (8000–10000 *g*) the cell suspension becomes quite viscous as a result of ruptured cells; microscopic examination shows irregular forms and spheres.

Similar changes could be followed microscopically when cells grown in 4.5 or 3.0 *M* salt are placed in salt concentrations below 3.0 *M*. As the concentration of salt decreases the cells pass through a succession of changes; they first bulge in the central or terminal portion of the rod, forming clubs, 'rabbit ears', and other irregular forms (Fig. 3); the bulge gradually increases producing a sphere 1–2 μ in diameter (average 1.6 μ) (Fig. 4), the rod portion simultaneously decreases in length leaving a ghost which eventually disappears. At a salt concentration of about 1.5 *M*, the spheres disappear and ghosts cannot be found.

When cells are subjected to a drastic osmotic shock, as when a drop of water is placed at the edge of a wet mount, cells near the edge of the cover slip swell slightly, then rupture, usually towards one end, and the cell contents flow out, without the formation of spheres. As might be expected, both the rapid and gradual change may be observed in the same preparation depending on the salt gradient. If the ruptured rods or spheres migrate towards the higher salt concentrations near the center of the mount, rod-shaped and spherical ghosts may be seen for a short time.

These observations suggest that under a drastic osmotic shock the cells rupture as a result of rapid swelling. With a more gradual lowering of the salt concentration, the cells undergo a gradual structural weakening which results, after a definite sequence of transitional forms, in the production of spheres.

Optical Density of Cells Suspended in Sodium Chloride Solutions

Freshly prepared suspensions of cells of *H. cutirubrum* in salt solutions show little change in optical density until the salt concentration is more than 0.5 moles below that of the growth medium (Fig. 7). However, in suspensions in salt solutions of lower concentrations, there is an increase in optical density until about 1.5 *M*; below this there is an abrupt decrease. The increase varies from about 25%, when cells grown in 3.0 *M* salt are used, to 80% with those grown in 4.5 *M* salt.

Microscopical examination indicates that as the salt concentration decreases and the optical density increases, there is an increase in the number of transition forms at the expense of rod forms (Table I). At the point of maximum optical density, the suspension consists mainly of transitional forms and spheres, and the increase in turbidity is no doubt the result of the increased

light scattering ability of the irregular forms. The abrupt decrease in optical density begins when all cells change to spheres, and continues as the spheres disappear. The salt concentration at which the abrupt decrease occurs is constant at any one time for cells grown in media containing 3.0 to 4.5 *M* salt. However, since the point of inflection changes from 1.5 *M* in freshly prepared suspensions to 2.5 *M* in suspensions 3 hours old, the changes are time dependent. The ability of the cells to produce colonies on agar decreases rather abruptly with the change to transition forms and spheres, and only about 60% of cells suspended in 2 *M* salt solution are viable after 1 hour although in this concentration the optical density is about maximal. In 1.0 *M* salt fewer than 1% of the cells are viable, although the optical density is still one third of the original value.

TABLE I

Optical density, viability, and morphology of cells of *H. cutirubrum* grown in 4.5 *M* salt medium after exposure for 1 hour to lower salt concentrations

NaCl conc. (moles)	Optical density (%)	Viable cells (%)	Morphology
4.5	100	100	Rods
3.5	108	100	Rods
3.0	121	100	Rods, sl. swollen, few transition forms
2.5	142	81	Rods and transition forms
2.0	155	62	Very few rods, equal numbers transition forms and spheres
1.5	122	21	Spheres
1.0	34	0.3	Spheres
0.5	0	0	No cells visible

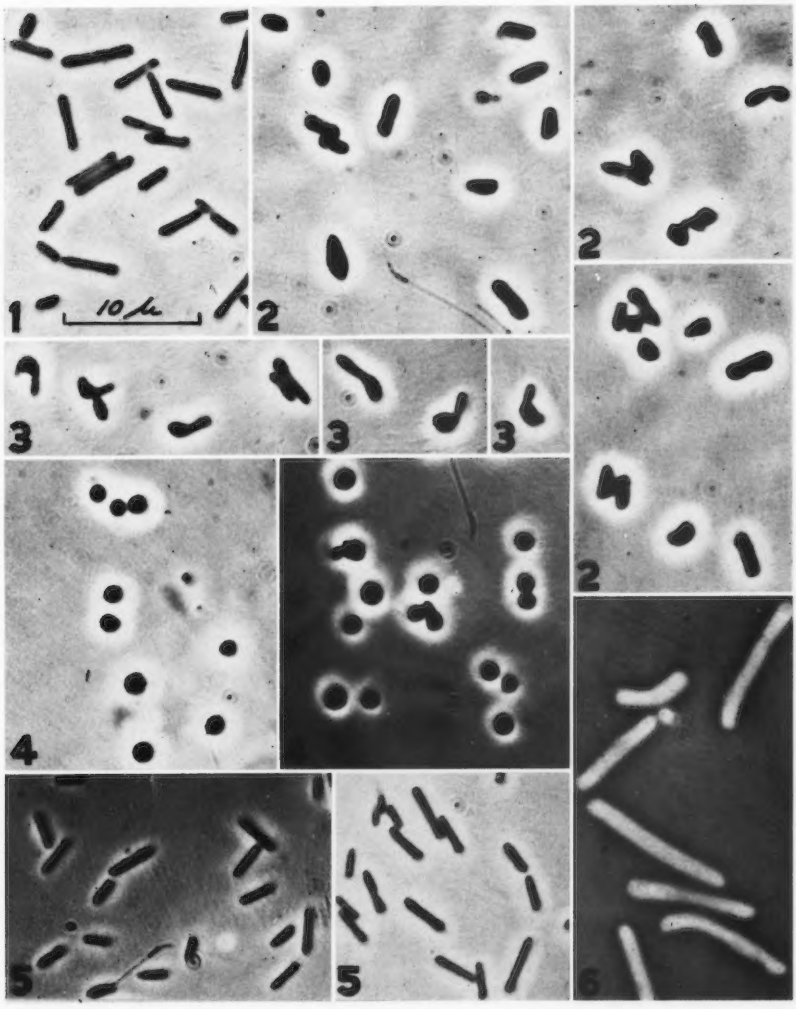
The change in optical density of suspensions is influenced by the temperature. An increase in temperature generally causes an increase in optical density except at salt concentrations below those giving maximum optical density when the reverse is true (Table II).

The increase in optical density of 4.5 *M* grown cells suspended in salt concentrations between 3.0 and 2.0 *M* is about 0.05 units and this is about

TABLE II

Effect of temperature on optical density ($\times 10^3$) of suspensions of *H. cutirubrum* held 4 hours at various salt concentrations

NaCl conc. (moles)	Temperature		
	5° C	25° C	44° C
5.0	113	132	143
4.5	110	132	144
4.0	115	144	150
3.5	139	162	195
3.0	145	174	218
2.5	190	198	224
2.0	200	222	196
1.5	175	135	106
1.0	22	32	20



FIGS. 1–6. Photomicrographs of cells of *H. cutirubrum*; magnification 2420×; phase contrast, dark M.

FIG. 1. Cells grown in 4.5 *M* salt. FIG. 2. Cells grown in 3.0 *M* salt. FIG. 3. Transition forms of cells grown in 4.5 *M* salt when placed in 2.5 *M* salt. FIG. 4. Spherical forms of cells grown in 4.5 *M* salt when placed in 1.5 *M* salt. FIG. 5. Cells grown in 4.5 *M* salt, treated with formaldehyde and suspended in 4.5 *M* salt. FIG. 6. As 5 but suspended in 0 5 *M* salt.

the same as the total increase in density of 3.0 *M* grown cells (Fig. 7). When cells grown in 4.5 *M* salt are held for 4 hours in 3.0 *M* salt and then resuspended in solutions of lower salt concentrations an increase in density is obtained similar to that with 3.0 *M* grown cells. Heavy suspensions of cells held in 4.5 and 3.0 *M* salt were adjusted so that, when diluted to the same extent in 4.5 *M*, salt suspensions of equal density were obtained. The suspension of cells held in 3.0 *M* salt contained 15% fewer cells, as determined by direct count, than the suspension of cells held in 4.5 *M* salt. Dilutions of the cells held in 4.5 and 3.0 *M* salt in 4.5, 3.0, and 2.0 *M* salt then gave optical densities (expressed as percentage of optical density in 4.5 *M* salt) of 100, 123, and 148 and of 100, 102, and 124, respectively. A progressive irreversible change evidently takes place as the salt concentration is reduced, and the optical density obtained is dependent on the morphological condition as well as the number of cells.

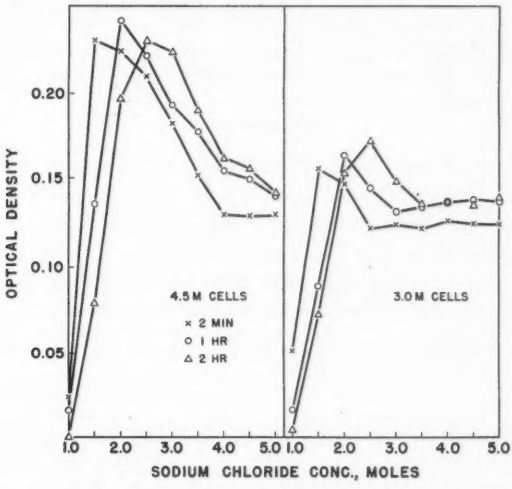


FIG. 7. Optical density of cells of *H. cutirubrum*, grown in media containing 4.5 and 3.0 *M* salt, when suspended in different concentrations of sodium chloride. Readings taken after 2 minutes and 1 and 5 hours.

Effect of Additional Iron in the Growth Medium on the Osmotic Sensitivity of Cells

The addition of iron to the medium accelerates the growth, increases the yield of cells, and allows growth in 2.4 *M* salt (9), suggesting that there might be a modification of the osmotic regulation in the cells of this organism. Cells grown in media containing 4.5 *M* salt, with and without added iron, were therefore tested in salt solutions of decreasing concentration (Fig. 8).

The curves obtained for cells grown in the absence of added iron are practically identical with those obtained previously (Fig. 7). Freshly prepared suspensions of cells grown in the presence of 10 p.p.m. added iron show little change in turbidity until a salt concentration of 1.0 *M* is reached (Fig. 8).

On standing, suspensions in 1.5 to 3.0 *M* salt increase in turbidity similarly to cells grown without iron, but never to as great an extent. Also, the inflection point of the turbidity curves of cells grown in iron remains at 1.5 *M* whereas the cells grown without iron begin to rupture in about 2.0 *M* salt after 3 hours.

Cells grown with iron retain their rod form at lower salt concentrations than those grown without iron. All cells eventually pass through the transitional phase to spheres as the salt concentration is lowered, but the cells grown in the presence of iron change much more slowly than those grown in its absence.

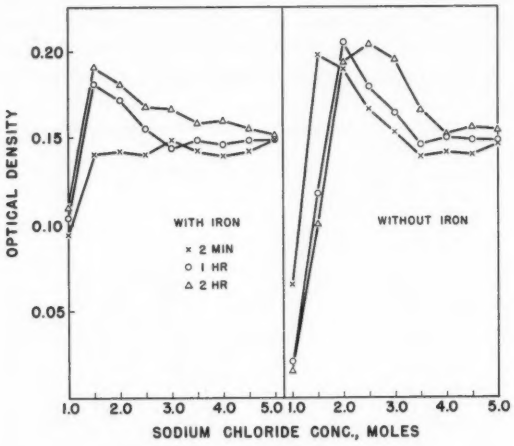


FIG. 8. Optical density in different salt concentrations of cells of *H. cutirubrum* grown in 4.5 *M* salt with and without added iron.

Response of Formaldehyde-treated Cells to Change in Salt Concentration

Cells of *H. cutirubrum* may be fixed in any of the described morphological shapes by treatment with 2% formaldehyde (see methods). However, the cells are not "fixed" in the conventional sense as they act as osmometers and swell and shrink as the osmotic pressure is increased or decreased. Furthermore, rods swell as rods and spheres as spheres. The various forms may increase in volume 10–14 times (Figs. 5 and 6) and few break, even in distilled water. Cells in water may measure 0.9–1.3 $\mu \times$ 3–8 μ whereas the original rods measured 0.5–0.7 $\mu \times$ 2–5 μ. The process is not altogether reversible, although swollen rods revert to almost their original size.

Cells grown in 4.5 *M* salt and treated with formaldehyde in the same concentration of salt were suspended in solutions of decreasing salt concentration and the effect on optical density determined (Fig. 9). In freshly prepared suspensions there is a barely perceptible increase in optical density between 5.0 and 2.0 *M* and no change in size can be detected microscopically. However, with time, cells suspended at salt concentrations of 3.0 *M* and less begin to

swell and become less distinct under the microscope. This is reflected in a decrease in optical density. This swelling and decrease in density is more apparent at high than at low temperatures.

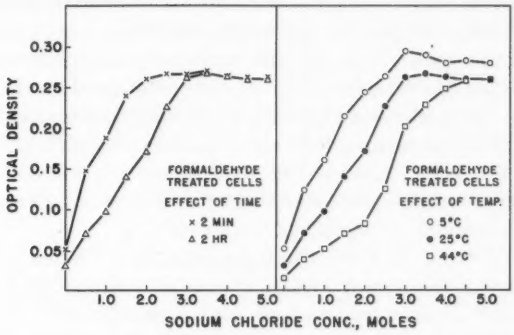


FIG. 9. Optical density of formaldehyde-treated cells of *H. cutirubrum* suspended in different concentrations of salt as influenced by time and temperature.

Discussion

The role of salt in the life of the red halophilic rods is clearly not solely osmotic but also involves the maintenance of their structural integrity. In fact, a high salt concentration is required to maintain the rigid structure of the cell wall of these organisms. This is in agreement with other studies (5), which have shown that the salt requirement is more specific than it would be if only a high osmotic pressure were required, and also with enzyme studies (3, 4), which showed that salt is required to maintain the fine structure of the enzyme protein.

The three halophilic rods used in this work have a definite rigid structure since cells grown in 4 to 5 *M* salt are regular slender rods which, when subjected to drastic osmotic shock, rupture leaving behind rod-shaped ghosts. These ghosts may be observed by phase contrast under certain conditions but disperse in low salt concentrations and as yet no way has been devised of collecting them. Most probably there is first a rapid ingress of water causing the cell to rupture, leaving a rod-shaped ghost which soon disappears.

If the salt concentration is reduced gradually the cells change from rods to transitional forms to spheres. At present it is not possible to say whether this change in morphology is the result of loss or denaturation of the cell wall material responsible for the rigidity and rod shape. The denaturation hypothesis seems plausible, especially when considered along with the behavior of these cells on heating and in the presence of other salts and protein denaturants as described in a subsequent paper (1). High concentrations of sodium chloride may be necessary to maintain protein structure, as suggested by Baxter (3) for halophilic enzymes, or to maintain an ionic balance in the protein or lipoprotein fraction of the cell wall. The lack of either rod-shaped or spherical ghosts in water or very low salt concentrations indicates a loss of

cell wall material, although as yet attempts to detect cell wall residues or components in the medium have failed. However, all possibilities have not been explored as yet. Possibly, the cell wall components are denatured at salt concentrations between 3.0 and 1.5 *M*, resulting in the change in morphology, and begin to disintegrate at lower salt concentrations.

It must be concluded that the cell walls of these organisms are unlike those of most other bacteria. Not only are they influenced by the ionic environment but even in high concentrations of salt they possess little mechanical strength since slight pressure on a cover slip, or centrifugation, is sufficient to produce spherical forms from many rods. If iron is added to the growth medium, the cells are most resistant to mechanical deformation and the changes in low salt concentrations take place more slowly.

When formaldehyde is added the rigid material is changed into an elastic membrane and the cells now act as osmometers, swelling and shrinking as the salt concentration is decreased or increased. Formaldehyde usually produces methylene bridges between amino groups and although this may impart a considerable degree of elasticity, formalin-treated cells have seldom been subjected to forces which would result in a volume increase of 10–14 times. Since formaldehyde-treated spheres also act as osmometers, formaldehyde confers the same elastic property to both spheroplast membrane and rigid cell wall. Fitz-James (7) found that protoplasts of *B. megaterium* fixed with osmium or formaldehyde would also swell and shrink with changes in osmotic pressure.

Mager *et al.* (8) and Avi Dor *et al.* (2) noted an increase in turbidity of suspensions of non-halophiles and moderate halophiles as the salt concentration of the suspending media was increased, followed by a return to the original turbidity with time. They associated the increase with a contraction of the cytoplasm with increased osmotic pressure followed by a return to normal as the cell adjusted to the increased salt concentration. The work with the extreme halophiles reported here has been mainly concerned with decreases in salt concentration but no increase in turbidity has been noted when cells grown in 3.0 *M* salt have been suspended in 4.0 and 4.5 *M* salt.

Christian (6) reported that *H. halobium* lysed at an external sodium chloride concentration which was a constant fraction (approximately 0.5) of the concentration in the growth medium. Furthermore, Christian observed a gradual decrease in turbidity as the salt concentration was decreased until the lysis point was reached. Although the present work was done mainly with *H. cutirubrum*, it has been repeated with *H. halobium* with the same results—an increase in turbidity as the salt concentration is decreased, the amount depending on the salt concentration of the growth medium, followed by lysis at 1.5 to 2.5 *M* regardless of salt concentration in the growth medium and depending on the time the turbidity readings are taken. Furthermore, the turbidity changes noted are accompanied by a definite sequence of changes in the morphology of the organisms. Christian believes the changes he noted gave some indication of the internal ionic concentration. We would relate the

changes described to changes in the cell wall and it is only with drastic changes in osmotic pressure that the ingress of water is sufficiently rapid to cause bursting of rod-shaped cells. With less drastic changes there is a rapid change in the cell wall and even with the spheres formed it is difficult to say definitely whether they burst as a result of water uptake or weakening of the spheroplast membrane. The changes noted here with changes in salt concentration are not accompanied by detectable changes in volume of cells, indicating a rapid adjustment to osmotic equilibrium. Formaldehyde-treated cells swell and shrink with changes in salt concentration indicating that the cells maintain a high internal osmotic pressure and that the formaldehyde-treated membrane is no longer permeable to the ions responsible for this pressure.

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THE IMPORTANCE OF INOCULUM STANDARDIZATION IN NUTRITIONAL EXPERIMENTS WITH FUNGI[1]

E. W. B. Ward and N. Colotelo

Abstract

Parent culture age, method of homogenation, and quantity of inoculum per culture flask were investigated for their influence on variability in yield in studies of the vitamin nutrition of a low-temperature basidiomycete. Age of parent cultures affected yield both in complete media and in basic (vitamin-free) media, in the latter presumably because of vitamin carry-over. Saline used as a suspending medium for homogenate preparation was found to be inhibitory when compared with water; the length of time of homogenizing, over the range tested, was without significant effect on either the mean yield or the variation between replicates. Variability between replicates was found to be inversely related to the quantity of inoculum per culture flask over a certain range, and yield tended to increase with increasing inoculum up to a limit.

The results emphasize the necessity of adopting a standard procedure for the preparation of inoculum if quantitatively reproducible results are to be obtained.

Introduction

The experiments described in this paper were initiated during shake culture studies of the vitamin requirements of the low-temperature basidiomycete (1) when variation in yield between replicate cultures and between experiments prevented detection of all but major differences in growth. Similar difficulties have been encountered by other investigators and they have shown how errors may be introduced by the inoculum used in seeding culture media. Taber and Vining (10) found that, with *Claviceps purpurea*, variation between replicates was inversely proportional to the quantity of inoculum used. Taber (9) showed that both the history and size of the inoculum influenced subsequent growth of *C. purpurea* and *Alternaria* sp. MacLeod (6, 7) found that yield of *Hirsutella gigantea* in shake culture was influenced by inoculum size and demonstrated the importance of nutrient carry-over with inoculum. Crasemann (3) and Hall (5) also correlated increase in yield with increase in inoculum.

In this paper a number of experiments are described which demonstrate the effect on subsequent growth of the age of the cultures used as inoculum source, the method of preparation of the inoculum, and the quantity of inoculum per culture flask.

Materials and Methods

The Isolate

The fungus used in these studies was isolated in 1948 from alfalfa plants at Lacombe, Alberta. Since then it has been maintained in laboratory culture and has also undergone several passages through host material in the field. It is one of a series of isolates similar to that obtained by Broadfoot and Cormack

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(1) that cause severe crown-rot of alfalfa and snow mold of grasses in Western Canada (2). As fruiting structures have not been discovered the taxonomic position of the fungus is unknown. However, its activity at rather low temperatures and the presence of typical basidiomycetous clamp connections have led to the use of the term "low-temperature basidiomycete" in previous publications. During the course of these experiments it was maintained on slants of M.Y.G. agar (see below) in test tubes at 10° C.

Media

Still cultures of the fungus were grown in 200-ml Erlenmeyer flasks on 25 ml of an organic medium composed of malt extract (Difco) 5 g, yeast extract (Difco) 5 g, D-glucose 15 g, and distilled water 1000 ml. This medium was solidified with 17.5 g agar (Difco–Bacto) for use in Petri dishes. It will be referred to subsequently as M.Y.G.

A basic synthetic medium was used in shake culture experiments: this does not support growth of the fungus without the addition of vitamins. It was made up as follows:

Major nutrients:	D-glucose	15.00 g
	l-asparagine	2.36 g
	KH_2PO_4	1.00 g
	$MgSO_4.7H_2O$	0.50 g
Minor nutrients:	$Fe_2(SO_4)_3$	0.715 mg
	$ZnSO_4.H_2O$	0.540 mg
	$Co(NO_3)_2.6H_2O$	0.245 mg
	$MnCl_2.4H_2O$	0.135 mg
	$H_2MoO_4.H_2O$	0.085 mg
	$CuSO_4.5H_2O$	0.195 mg
	$CaCl_2$	4.0 mg
N KOH to adjust pH to 6.0		
Distilled water to make 1 liter		

The minor nutrients were made up in a stock solution and the inclusion of 1 ml of this in 1 liter of medium gave the required concentration.

The following vitamins were included in the above to give a medium adequate for growth:

Thiamine hydrochloride	100 μg
Calcium pantothenate	100 μg
Pyridoxine hydrochloride	100 μg
d-Biotin	10 μg
i-Inositol	5 mg

The vitamins were added to the medium from stock solutions 1000 times the required concentrations. The basic medium with added vitamins will be referred to as a "complete medium". Inorganic chemicals were of reagent grade; biochemicals were of the highest grade commercially available.

Experiments with synthetic media were carried out by the shake culture method, using 200-ml Erlenmeyer flasks containing 50 ml medium. The flasks were closed with loose aluminum foil covers. All media were sterilized by autoclaving at 121° C for 9 minutes. Glassware was cleaned by washing with detergent solution, rinsing with tap water, rinsing with chromic acid, rinsing with tap water (6 times), and finally with distilled water (6 times).

Preparation of Inoculum

The procedure finally adopted, and outlined here, was based in part on the findings to be described subsequently in this paper. It had two main objectives (*a*) to provide inoculum in which vitamin carry-over was reduced to a minimum, and (*b*) to provide a standard method giving optimum growth on the complete medium and reducing variation that, as will be shown later, can be introduced by small differences in method.

There were three main steps, as follows:

Petri dish cultures.—Petri dishes containing 15 ml of MYG agar were inoculated with mycelium from stock cultures and incubated at 15° C. The growing periphery of the culture was regarded as a uniform and reproducible type of growth.

Still cultures.—To increase the amount of mycelium and provide it in a form suitable for homogenizing, flasks containing 25 ml M.Y.G. liquid medium were inoculated with plugs 5 mm in diameter cut from the periphery of Petri dish cultures, and incubated at 15° C for 24 days.

Basic medium shake cultures.—The carry-over of vitamins with mycelium from the still cultures was too high, even after repeated washing, to permit its use directly as inoculum for vitamin studies. Consequently it was homogenized and used to inoculate shake culture flasks containing the basic medium, where growth was dependent on the vitamin carry-over from the still cultures. After 12 days' incubation the mycelium was homogenized to provide the final inoculum preparation.

The preparation of homogenates was as follows:

(*a*) *From still cultures.*—After removing the medium aseptically, the mycelium from 24-day-old still cultures was twice washed with 2 volumes of approximately 100 ml sterile distilled water. It was then suspended in sterile distilled water and homogenized for 90 seconds in a sterile semimicro Monel metal container of a Waring blendor assembly. An aliquot of the homogenate was transferred by pipette to a sterile 125-ml Erlenmeyer flask fitted with a gauze-wrapped cotton plug over which an aluminum foil cover was placed. The flask and contents were stored at 2° C until required. Storage was seldom in excess of 6 hours; after longer periods coagulation tended to occur, necessitating additional homogenation. The dry weight of the homogenate was determined from three 5-ml samples which were filtered on previously dried and weighed filter papers, washed thoroughly with distilled water, and dried at 90° C until successive weighings were identical. The homogenate was then adjusted with sterile distilled water to a dry weight value of 2 mg per ml.

(*b*) *From basic medium shake cultures.*—The medium from the shake culture was removed after centrifuging for 4 minutes at 10,000 r.p.m. in a Servall Superspeed centrifuge. The mycelial pellets were then resuspended in sterile distilled water and homogenized, etc., as above.

One milliliter of a homogenate (dry weight 2 mg) was used to inoculate shake culture flasks using a 2-ml syringe fitted with a 3½-in. 15-gauge hypodermic needle.

The container from the Waring blendor was cleaned in detergent and rinsed thoroughly with tap water. It was sterilized by rinsing with a 50% "Perfex"* solution, followed by four changes of sterile distilled water. Nylon centrifuge tubes and caps were washed in detergent, thoroughly rinsed with tap water followed by distilled water; they were sterilized by autoclaving.

Incubation and Estimation of Growth

Unless otherwise indicated shake cultures were incubated in the dark at 15° C for 12 days on a Gyrotary† shaker with a radius of motion of 1 inch operating at approximately 275 r.p.m. Still cultures were incubated at 15° C in a constant temperature cabinet and received light of low intensity, indirectly, from a northern window. Growth was determined as the mean dry weight of four replicate cultures. The mycelium was harvested on previously dried and weighed filter papers, repeatedly washed with distilled water, and dried at 90° C for 24 hours.

Experimental Results

Effect of Culture Age on Growth from Homogenized Mycelium

The proportion of active to resting or senescent mycelium, nutrient availability, and accumulation of inhibitory or staling products are obvious variables that may be correlated with the age of a culture. Inoculum, consisting of homogenized mycelium, may therefore be affected by the age of the parent culture. A series of experiments is described below, which indicate the importance of the age of the still cultures and basic medium shake cultures used in the inoculum preparation procedure.

To determine the effectiveness of final inocula from cultures of different ages, use was made of the fact that the fungus requires a vitamin supplement for growth. Using (*a*) still cultures and (*b*) basic medium shake cultures of various ages, growth from final inocula was compared on both the complete and basic media. Growth on the former would be influenced by the proportion of active mycelium and possible inhibitors in the inoculum, while that on the latter would be proportional also to vitamin carry-over. In addition, the effect of variation in the age of still cultures on growth in the basic medium shake culture stage was determined.

Variation in Still Culture Age

(*a*) *Growth of still cultures.*—A time–growth curve for still cultures was obtained by harvesting four replicate flasks at 4-day intervals from 8 to 40 days after inoculation. The mean dry weights obtained are plotted in Fig. 1. After a lag of approximately 8 days, growth increased rapidly until 20 days, and was then presumably counterbalanced by autolysis.

(*b*) *Effect of still culture age on growth in basic medium shake culture.*—Basic medium shake cultures were inoculated with homogenates (1.74 mg/ml) of still cultures aged 12 to 40 days and incubated for 12 days. The mean dry

*Sodium hypochlorite.
†New Brunswick Scientific Co., New Brunswick, N.J.

weights of each set of four replicate cultures were determined and the values plotted as (*b*) in Fig. 1. Growth from the 12- and 16-day cultures was high, indicating a heavy carry-over of vitamins. The 20-day and older material gave much less growth, coinciding with the reduction in growth in still culture at this age Fig. 1(*a*). This may indicate limitation in vitamin carry-over, increase in inhibitors, or decrease in proportion of active mycelium.

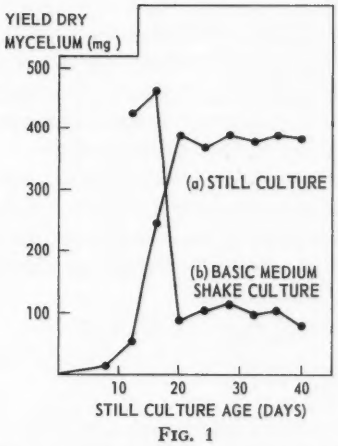

FIG. 1

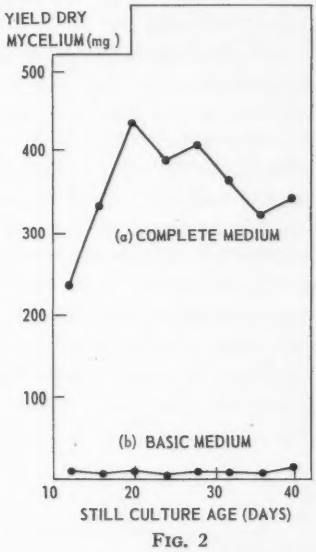

FIG. 2

FIG. 1. (*a*) Growth of still cultures and (*b*) the influence of still culture age on yield in basic medium (shake culture, 12 days' growth).

FIG. 2. Effect of still culture age on yield from the final inoculum on the complete and basic media (shake culture, 12 days' growth).

(*c*) *Effect of still culture age on growth from final inoculum.*—Four replicate flasks each of the complete medium and the basic medium were inoculated with the final inoculum (1.59 mg/ml) prepared from the above 12-day-old basic medium shake cultures that had originated from still cultures aged 12 to 40 days. The mean dry weights obtained after 12 days' growth are indicated in Fig. 2. From curve (*b*) it is apparent that carry-over of vitamins with the final inoculum was insignificant irrespective of the age of the original still culture. On the complete medium, however (Fig. 2(*a*)), growth from the different inoculum preparations varied considerably. Highest yields were obtained in cultures derived originally from 20- to 28-day-old still cultures, and the data indicate that some factor or factors in younger and older stil cultures persist through the basic medium shake culture stage and bring about a reduction in effectiveness of the final inoculum preparation.

Variation in Basic Medium Shake Culture Age

(*a*) *Growth of basic medium shake cultures.*—Basic medium shake cultures were established using homogenates (2 mg/ml) of 24-day-old still cultures and were harvested at 2-day intervals from 4 to 20 days after inoculation. The mean dry weights of four replicate cultures for each age are recorded in Fig. 3. In this experiment vitamin carry-over, apparently, was sufficient to permit rapid growth up to 12 days; growth ceased after 16 days.

(*b*) *Effect of basic medium shake culture age on growth from final inoculum.*—Final inocula (2 mg/ml) prepared from basic medium shake cultures aged 8 to 20 days, were used to inoculate four replicate flasks each of the complete and basic media. After 12 days' incubation mean dry weights were determined and are recorded in Fig. 4. Carry-over of vitamins from the 8- and 10-day-old cultures was appreciable (Fig. 4(*b*)) but from older material it was consistently low. On the complete medium (Fig. 4(*a*)) optimum growth resulted from homogenates of 12-day-old cultures, while younger and older sources gave significantly lower yields. The peak with 12-day cultures probably corresponds to the period of maximum growth (Fig. 3) in the basic medium shake culture stage, and the decline with inoculum prepared from older cultures could be due to a lower proportion of active mycelium, an increase in inhibitory factors, or both. The reduced yields obtained using the 8- and 10-day-old cultures may indicate the temporary development of a factor inhibitory to growth in

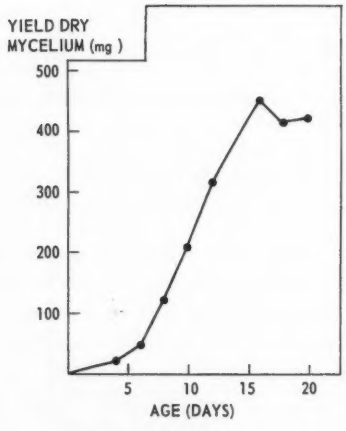

FIG. 3

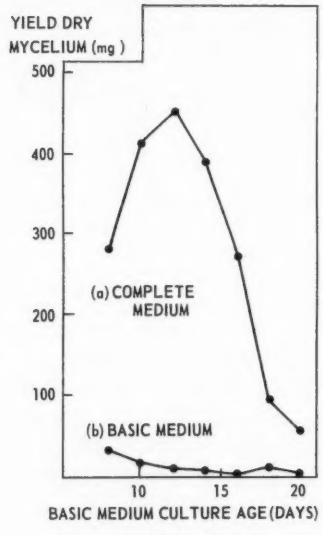

FIG. 4

FIG. 3. Growth of basic medium shake cultures.
FIG. 4. Effect of basic medium shake culture age on yield from the final inoculum in the complete and basic medium shake culture (12 days' growth).

the presence of the constituents of the complete medium. This situation is possibly comparable to that illustrated in Fig. 2(*a*), using inoculum derived originally from young still cultures.

Homogenation of Mycelium

Suspending Solution

Initially saline was used as a suspending solution for the preparation of homogenates. However, satisfactory growth occurred only when quantities of 15–20 mg dry weight were used to inoculate cultures, and this resulted in excessive nutrient carry-over from parent cultures. A report by Taber and Vining (11), that alkaloid production by *Claviceps purpurea* was less from cultures initiated with saline homogenates than with water homogenates, suggested that other suspending solutions might permit the preparation of more active inoculum. Accordingly, a number of experiments were carried out to compare growth from homogenates prepared with various solutions and with water. Highest yields were obtained from the water suspended homogenates, and in addition optimum growth occurred from smaller quantities. At these lower inoculum levels the toxic effect of the saline was even more apparent.

The results of an experiment comparing growth on the complete medium from final inocula prepared in 0.15 *M* KCl, 0.15 *M* NaCl, or sterile distilled water are summarized in Table I. In addition, equivalent quantities of KCl and NaCl were added to separate series of cultures started from homogenates prepared with water, and the data indicate that the salts were toxic directly to the homogenates.

TABLE I

Influence of suspending solution on growth from homogenized mycelium*

Suspending solution	Yield dry mycelium (mg)
H_2O	455.1 ± 32.7
KCl 0.15 *M*	23.7 ± 9.5
NaCl 0.15 *M*	5.0 ± 1.3
H_2O, 1 ml 0.15 *M* KCl added to culture flask	390.5 ± 42.3
H_2O, 1 ml 0.15 *M* NaCl added to culture flask	425.5 ± 19.7

*Dry weight adjusted to 2 mg/ml.

Length of Time of Homogenation

The possibility was considered that certain properties of the inoculum might be influenced by the extent of homogenation. Brief homogenation might give particles variable in size and few in number while more particles with greater uniformity, and possible loss of viability, might result from longer treatments. The extent of homogenation could, therefore, influence the yield from inoculum in nutritional studies.

Two experiments were carried out to determine the effect of length of time of homogenation of inoculum on yield, and the results obtained are recorded

in Table II. From these data it appears that under the conditions of these experiments length of time of homogenation had no consistent effect on yield.

TABLE II

Yield from inoculum* homogenized for various lengths of time

Experiment 1		Experiment 2	
Time (seconds)	Yield dry mycelium (mg)	Time (seconds)	Yield dry mycelium (mg)
30	356.7 ± 9.1	10	461.0 ± 49.6
90	357.4 ± 14.1	20	426.0 ± 40.8
180	351.8 ± 27.6	30	404.6 ± 52.0
270	367.7 ± 20.9	45	411.4 ± 8.6
360	341.8 ± 36.2	90	423.9 ± 29.7

*Inoculum 2 mg/ml, basic medium shake culture used for inoculum, 9 days old in experiment 1, 12 days old in experiment 2.

Effect of Variation in Quantity of Inoculum

If yield of mycelium is related to the amount of inoculum used, quantitative errors in the addition of inoculum to culture flasks or lack of uniformity in homogenates are obvious sources of variability in nutritional studies. Information on the influence of the quantity of inoculum on variation, and on the amount of inoculum giving optimum yield under the particular conditions of an experiment would therefore be of value. Foster (4) recommended the use of high inoculum levels (5–10% by volume) for maximum efficiency, and Taber and Vining (10) and MacLeod (6) reported that minimum variation between replicates occurred with a large inoculum. However, nutrient carry-over is proportional to the amount of inoculum, and as either excess variation or nutrient carry-over may invalidate an experiment, Taber (9) suggested that the smallest quantity of inoculum giving a coefficient of variation not greater than 10% be selected.

Experiments were carried out with the low-temperature basidiomycete to determine the effect of quantity of inoculum on mean yield and variation between replicates. Homogenates of 12-day-old basic medium shake cultures were prepared and adjusted to give dry weight values from 0.05 to 3.0 mg per ml. One milliliter of a homogenate was used to inoculate four replicate flasks containing the complete medium. The cultures which developed were harvested after 12 days and the dry weights determined. The results, together with the coefficients of variation, are given in Fig. 5.

Yield of mycelium increased quite rapidly with inoculum increments up to the 1.25-mg level, but, above this, additional inoculum produced comparatively smaller increases in yield. The coefficient of variation, fairly constant with 3.0, 2.0, 1.5, and 1.25 mg of inoculum, rose rapidly with further decreases in inoculum until the 0.1 and 0.05 mg levels were reached. This might be anticipated from the shape of the yield curve (Fig. 5). Any quantitative error in transfer of inoculum below the 1.25-mg level would have a greater effect on yield, and hence on variation, than a similar error at higher levels.

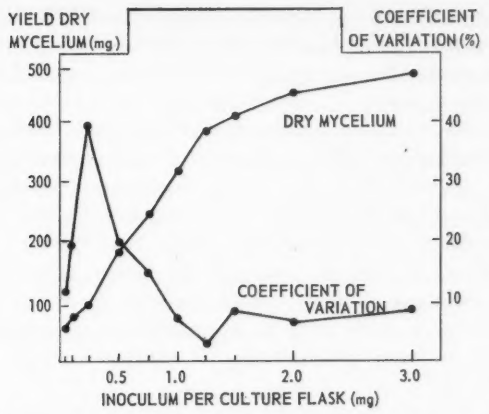

FIG. 5. Effect of quantity of inoculum on yield and variation in the complete medium (shake culture, 12 days' growth).

It may be concluded from the yield curve that in cultures inoculated with 1.25 mg or less, the quantity of inoculum was the main factor limiting growth during the incubation period used. Above this inoculum level the situation changed to one in which inoculum was in excess and nutrient and other environmental factors became growth-limiting. The optimum inoculum level for nutritional studies should logically be chosen from this part of the curve, for it is only here that quantitative variation in inoculum ceases to be a major factor; with other environmental factors being constant, nutrient supply becomes the main criterion of growth. The limitation of growth by nutrient supply at high inoculum levels and its enhancement with a higher nutrient–inoculum ratio at lower levels is emphasized when the values of Fig. 5 are computed on a growth per mg basis as in Table III. It is apparent from these figures, however, that the situation is by no means simple and possibly factors additional to the nutrient–inoculum ratio influence growth at least down to the 0.25 mg inoculum level, for only below this level is there any indication of proportionality between quantity of inoculum and growth. Further study would be required to establish whether the reduction in the coefficient of variation that occurred with 0.05 and 0.10 mg of inoculum is of significance (Fig. 5).

Discussion

The effect on yield of the age of the cultures used as inoculum source may be related to the extent of carry-over of nutrients, or inhibitory staling products, or to the proportion of active mycelium in the inoculum. Although it was possible to demonstrate vitamin carry-over (Fig. 1(*b*), Fig. 3, Fig. 4(*b*)) it could not be determined from these experiments which of the other two factors was operating in complete media, when less than optimum growth was obtained (Fig. 2(*a*), Fig. 4(*a*)). Reduction in growth with inoculum from young cultures, e.g. the first two ages used in Figs. 2(*a*) and 4(*a*), suggests

the formation of some type of inhibitor as the most likely explanation. With inoculum from older cultures both inhibitors and a lower proportion of active mycelium may operate in reducing growth. Another possibility which should not be completely ignored is that of selection of physiological strains as a result of the different cultural conditions that occur with increasing age. The value of washing inoculum and growing it on a medium similar to the one used in the experiment is emphasized by these considerations. The age effect, although not eliminated, can be controlled by the use of standard incubation periods and conditions for growth of inoculum.

On the basis of the results obtained the culture ages described under "Methods" would seem to be reasonable ones to select, but only a few age combinations of the various cultures were tested in the experiments and it is probable that an even greater range of variation would be revealed by further experiments.

The toxic effect of KCl and NaCl was surprising as saline has been found a satisfactory suspending medium by other investigators (6, 10). The pH of both solutions is close to 6.0, and as this was the pH of the complete medium it is unlikely that pH was the factor involved. Tests carried out in which glucose at concentrations up to 0.25 *M* was substituted for NaCl gave no indication of toxicity and this suggests that osmotic effects were not responsible.

The data of Savage and Vander Brook (8) do not indicate any striking differences in penicillin yield from cultures derived from inoculum that was homogenized from 0.5 to 10 minutes. This agrees well with the results reported in this paper. It suggests that, with the apparatus used, this factor can be ignored as a source of variation, and this is important because inconsistency in homogenation can hardly be avoided unless care is taken to ensure that equal volumes are homogenized on all occasions. However, 2 mg of inoculum was used and it was demonstrated subsequently (Fig. 5) that at this level small variation in the amount of inoculum was without major effect on yield. Hence it is possible that the experiments did not reveal small differences in homogeneity or in viability of inoculum produced by different periods of homogenation.

The results reported here on the effect of quantity of inoculum on yield and variation between replicates are in general agreement with those of other investigators (3, 5, 6, 7, 9, 10). Yield increased with increased inoculum until nutrient and other environmental factors became limiting. With such high inoculum: nutrient ratios, variation in quantity of inoculum had less influence on yield. At lower inoculum levels, nutrients ceased to be a limiting factor during the period of incubation used, and the inoculum concentration became critical. These results permit a satisfactory explanation of the increased variation observed with low inoculum levels (6, 7, 10), where any lack of homogeneity, or error in inoculum transfer will have, proportionately, a much greater effect on yield. Hall (5) reported that although the yield of mycelium of *Phytophthora infestans* in still culture increased with inoculum

size, some toxic factor in autoclaved media, correlated with nitrogen level, caused relatively stronger inhibition of growth at low than at high inoculum levels. The possibility that factors of this type might occur with particular nutrients could lead to erroneous conclusions in nutritional experiments if small amounts of inoculum are used. Taber (9) and MacLeod (7) also consider that differences in inoculum level may give qualitative differences in nutrient utilization as well as quantitative effects on yield. However, treatment of the results of these investigators in the manner of Table III indicates that in many cases, in spite of the obvious differences in total yield, utilization of nutrients by a small inoculum as indicated by growth rate, is usually as high as and frequently higher than by a large inoculum, and their results in this respect are probably not conclusive.

TABLE III

Yield of mycelium from various quantities of inoculum calculated as the growth per mg of inoculum (data from Fig. 5)

Inoculum, mg	0.05	0.10	0.25	0.50	0.75	1.00	1.25	1.50	2.00	3.00
Calculated yield per mg	1152	746	378	363	325	314	307	271	225	160

As the inoculum level is decreased the number of particles in culture flasks becomes correspondingly less, and their morphology changes from minute amorphous fragments consisting of a few branching hyphae to large round balls several millimeters in diameter. This was pointed out by Foster (4), and it is evident that if the quantity of inoculum is sufficiently low the limiting factor in growth will be the size to which the particles can develop. This is obviously an additional complicating factor in the growth of filamentous fungi in shake culture, which does not occur with unicellular forms, and may contribute to the lack of consistency found with small amounts of inoculum.

Two main factors, age of cultures used for preparation of inoculum, and quantity of inoculum, have thus been demonstrated to influence yield and hence variation either within or between experiments. These two factors were considered separately, but it is obvious that many different combinations of them might be used. In addition, many authors use different experimental conditions such as quantity of medium, flask size, shaker speed, etc., and all can be expected to influence results at least quantitatively. All these considerations indicate that without the adoption of carefully standardized procedures for the production and use of inoculum in nutritional experiments with fungi, quantitatively reproducible results are more likely to be the exception than the rule.

Acknowledgment

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GROWTH REQUIREMENTS OF 94 STRAINS OF THERMOPHILIC BACILLI[1]

H. Baker, O. Frank, I. Pasher, B. Black, S. H. Hutner, and H. Sobotka

Abstract

The minimal nutritional requirements of 94 thermophilic strains of the species *Bacillus stearothermophilus* and *B. coagulans*, grown at 55° C, were determined. Most strains had an absolute methionine requirement. Some strains synthesized methionine by means of a methyl donor (choline, betaine), a methyl acceptor (homocystine), and a methyl activator (vitamin B_{12}). The effects of temperature as inducing variations in nutritional requirements are briefly discussed.

Introduction

Knowledge of the metabolism of aerobic spore-forming thermophilic bacilli (defined here as growing above 55° C) provides insight into the determinants of the heat stability of protoplasm, especially of the proteins and enzymes. As more about the nutrition of the thermophilic bacilli became known (2, 3, 4, 8), chemically defined media were developed for investigating hitherto inaccessible metabolic pathways. Recent reviews (2, 10) stress the dynamic nature of thermophily, i.e. in growth rapid synthesis and repair keep pace with rapid destruction. The minimal requirements of some strains of *Bacillus coagulans* and *B. stearothermophilus* have been described (8, 9). Determination is complicated by the findings that in many cases the minimal requirements decrease with increasing temperatures; sometimes the reverse holds.

The taxonomy of the aerobic spore-forming bacilli is treated critically and comprehensively by Smith *et al.* (21). Thermophilic bacilli fall into two well-defined species: (*a*) *B. coagulans* (nearly all strains grow between 33 and 60° C, none above 60° C); and (*b*) *B. stearothermophilus* (growing mostly between 37 and 65° C, with some able to grow above 70° C). The work reported here was carried out with authentic strains of these two species, comprising 26 strains of *B. coagulans* and 68 strains of *B. stearothermophilus* as identified and supplied by Dr. R. E. Gordon of Rutgers University and the National Canners Association, Washington, D.C.

Methods

The chemically defined basal medium (Table I) contains a non-metabolizable chelating agent, nitrilotriacetic acid, and non-metalizable buffering agents, pyromellitic acid and triethanolamine. A metals supplement, added as dry mix (6), provides Fe, 0.2 mg (as $Fe(NH_4)_2(SO_4)_2.6H_2O$); Zn, 0.1 mg

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(as $ZnSO_4.7H_2O$); Mn, 0.05 mg (as $MnSO_4.H_2O$); Cu, 0.008 mg (as $CuSO_4.5H_2O$); Co, 0.01 mg (as $CoSO_4.7H_2O$); B, 0.01 mg (as H_3BO_3); Mo, 0.005 mg (as $(NH_4)_6Mo_7O_{24}.4H_2O$); V, 0.001 mg (as $Na_3VO_4.16H_2O$), when added as designated.

Preliminary experiments showed glycerol, lactate, and succinic acid to be choice substrates; asparagine was a good N-source. Commerical Na glycerophosphate (a mixture of the α- and β-isomers) served as a phosphate source; it was preferred to inorganic phosphate because of the greater solubility of its salts with Ca, Mg, and Fe. Addition of inorganic phosphate to the medium gave rise to precipitation.

The pH of the medium was adjusted to 6.8–7.2 with Quadrol (N,N,N′,N″-tetrakis (2-hydroxypropyl) ethylenediamine; Wyandotte Chemicals Corporation, Wyandotte, Michigan), rather than with potassium or sodium hydroxide, to minimize increase in osmotic pressure. The medium was stored double strength (twice the concentration listed in Table I) at 4° C with volatile preservative (15).

TABLE I

Basal medium for screening growth requirements of thermophiles

Constituent	Amount (mg/100 ml)*
Nitrilotriacetic acid	20.0
DL-Asparagine H_2O	400.0
Glycerol (w/v)	4000.0
$MgSO_4.7H_2O$	80.0
Metals mix†	2.1
Ca (as Cl)	5.0
KNO_3	50.0
K_2PO_4	10.0
Sodium glycerophosphate. $5H_2O$	600.0
Succinic acid	200.0
Pyromellitic acid	250.0
Triethanolamine (w/v)	500.0
DL-Lactic acid (w/v)	200.0

NOTE: pH 6.8–7.2.
*Distilled water to 100 ml.
†See methods.

Cultures were maintained on beef extract (Difco) 0.5 g; Polypeptone (Baltimore Biological Laboratory) 0.5 g; agar 2.0 g; distilled water to 100 ml. Stock cultures were incubated at 55° C for 24 hours and stored at 4° C. Transfers were made every 4 to 6 weeks. The basic techniques have been outlined (4, 5). For experiments, fresh 24-hour cultures were used; a loopful of the culture was suspended in 25 ml of basal medium. A drop of this suspension served as inoculum. Experimental cultures were incubated at 55° C $\pm$ 0.1° C.

The "complete" medium used for identifying minimal requirements contained 17 amino acids, 17 vitamins, 14 purines, and pyrimidines, including free bases, nucleosides, and nucleotides. All amino acids, vitamins, purines, and pyrimidines were obtained chromatographically pure from the California Corporation for Biochemical Research, Los Angeles, California. The "complete" medium was divided to detect requirements. A mixture of amino acids,

vitamins, purines, pyrimidines, beef extract, yeast extract, and a tryptic digest of casein (Trypticase, Baltimore Biological Laboratory) was used to imitate natural complex materials in testing the adequacy of the chemically defined medium.

TABLE II

Nutritional requirements for 68 strains of *B. stearothermophilus*

Organism	Methionine requirement	Other requirement	Stimulation
B. stearothermophilus Nos. 16, 19, 20, 30, 44, 61, 73, 80, 85, 106, 110, 137, 149, 152, 153, 158, 159, 164, 188, 189, 197, 1503N, 4578	+	−	−
27, 74, 75, 76	+	−	Glutamic acid
35, 39, 4259	+	−	Arginine
79, 150, 155	+	−	Threonine
194, 196	+	−	Folic acid
1	+	−	Lysine, arginine
4	+	−	Thiamine
29	+	−	Threonine, arginine, tryptophan, lysine, glycine
132	+	−	Histidine, arginine, aspartic acid
147	+	−	Valine
165	+	−	Threonine, arginine, histidine
166	+	−	Arginine, thiamine
195	+	−	Glutamic acid, folic, B_{12}
Agar liquefier	+	−	Threonine, lysine
108	+	Threonine	−
117	+	Arginine, glutamic acid	−
148	+	Tryptophan, threonine	−
168	+	Arginine	−
18	+	Folic acid	Thiamine, biotin
33	+	Thiamine, pyroxidine	B_{12}
39	+	Thiamine, biotin	Folic acid
127	+	Lysine	Folic, B_{12}
141	+	Glutamic acid	Alanine
154	+	Threonine	Thiamine, biotin
160	+	Tryptophan	Histidine
1102	+	Lysine, histidine	Folic, B_{12}
32	−	Alanine, proline	−
90	−	Biotin, nicotinic acid	−
107	−	Threonine, tryptophan	−
109	−	Inositol, riboflavin, thioctic acid	−
167	−	Biotin, thiamine, nicotinic acid	−
1356	−	B_{12}, quanine, cytidine	−
31	−	Glutamic acid	Arginine
63, 70	−	Glutamic acid	Arginine, histidine
67	−	Glutamic acid, histidine, biotin, thiamine, pyridoxamine	Nicotinic acid
72	−	Glutamic acid	Histidine
89	−	Pantothenic acid	Thioctic acid

Results

The organisms show four main nutritional patterns: those requiring (*a*) methionine only, (*b*) methionine plus other metabolites, (*c*) methionine, but clearly stimulated by other factors, and (*d*) not requiring methionine (Table II, III).

Methionine is interchangeable with vitamin B_{12} for growing *B. stearothermophilus* strains No. 158 and 1503-N and for *B. coagulans* No. 4, 15, 27-6, 710, 712, 831, 3084, 71924, and B_1.

A typical growth curve for a methionine requirer is given in Table IV. Ethionine was used as an antagonist to exaggerate the methionine requirement; the inhibition index is approximately 10. No inorganic or other organic sulphur sources satisfied the methionine requirement.

TABLE III

Nutritional requirements of 26 strains of *B. coagulans*

Organism	Methionine requirement	Other requirement	Stimulation
B. coagulans Nos. B_1, N15, 29-1, 43P, 714, 716, 831, 3084, C2253, F, 71924	+	−	−
4	+	−	Nicotinic acid
15	+	−	Histidine, pyridoxamine, thymine, PABA
27-2	+	−	Aspartic acid
27-6	+	−	Xanthine
27-8	+	−	Biotin, nicotinic acid
32G	+	−	Isoleucine, proline
57G	+	−	Thiamine, biotin
710	+	−	Alanine, glutamic acid
712	+	−	Glycine
821	+	−	Choline
27-5	−	Glutamic acid, histidine	−
2	−	Glutamic acid	Folic acid, B_{12}
139	−	Glutamic acid, histidine	Nicotinic acid
713	−	Glutamic acid, alanine	Thiamine
4167	−	Glutamic acid	Lysine, histidine

TABLE IV

A typical methionine growth curve using *B. coagulans* 32G*

DL-Methionine concentration (μg/ml)	Ethionine concentration (μg/ml)			
	0	300	1000	3000
0	0	0	0	0
0.1	0.08	0	0	0
1.0	0.16	0	0	0
10.0	0.42	0	0	0
100.0	0.54	0.52	0.34	0
1000.0	0.68	0.62	0.54	0

*Basal medium supplemented with DL-isoleucine and L-proline. Growth is measured in optical density units with a Welch Densichron. An optical density of 1.0 yields 0.55 g of dried bacilli per liter.

Because most strains, especially *B. coagulans*, require methionine, transmethylation reactions in these species were studied (Table V). In some strains homocystine alone substitutes for methionine; most need a methyl donor, a methyl acceptor, and B_{12} as a methyl activator.

TABLE V

Substitution for methionine in *B. coagulans*

Substituent for methionine	Strain No.										
	4 32G 57G 831	27-2 27-8 43P 710 714	15 712	29-1	27-6	N15	B1	F	716	821	C2253 71924
None	+										
Homocystine		+				+			+		+
Cystine, choline, betaine, B_{12}		+	+			+		+		+	
Homocystine, B_{12}, choline, betaine			+	+			+	+		+	
Homocystine, choline					+						
Cystathione						+					
Homoserine, B_{12}, cystine, choline							+	+			+
Glycine, B_{12}, serine								+	+		
Cystine									+		
Cystathione, B_{12}										+	
Homoserine, B_{12}, cystine										+	

Discussion

Because synthesis and cell destruction during growth at 55° C is rapid, culture media which permit essential syntheses at the highest possible rate are necessary. The relatively simple defined medium described here (Table I), is adequate despite its simplicity. When basal medium plus the essential nutrient requirement listed (Table II) is supplemented with complex material e.g. yeast autolysate, beef extract, casein hydrolyzate, etc., growth does not increase more than 0.2 optical density units (O.D.). This medium is suitable for mass cultures, thus it could facilitate further studies on the chemistry of thermophilic bacilli (22), as compared to mesophiles.

This investigation was only a survey of growth requirements; an exhaustive analysis of the various nutritional patterns or kinetics was not attempted. Oxygen supply probably limits growth of these cultures; oxygen is one-half as soluble at 55° C as at 30° C (1, 2), and thus, in the technique here described, media in shallow layers were necessary to insure proper aeration.

The nutritional requirements were strikingly uniform in that most strains require methionine. Such results are in agreement with Campbell and Williams (8) and Bhat and Bilimoria (7). O'Brien and Campbell (18) afterwards showed that minimal requirements for fermentation, outgrowth of spores,

and vegetative cell requirements for *B. stearothermophilus* and *B. coagulans* included methionine. Studies by Campbell and Sniff (9) showed that folic acid is required by *B. coagulans* grown at 45° C. At 55° C, the influence of folic acid in methylations (14, 16) seems to be lost as shown by the absolute requirement for methionine seen here (Table II, III). The diversity of the nutritional requirements encountered with mesophilic aerobic spore-forming bacteria (17, 18) suggests that mesophiles are nutritionally far more heterogeneous than thermophilic bacilli. Consequently the absolute requirements listed in the present paper may not be valid at temperatures other than 55° C.

The interchangeability between methionine and B_{12}, mentioned earlier, fits the pattern shown by the B_{12} or methionine-requiring mutant *Escherichia coli* 113-3, which is widely used as an assay organism for cobalamins. Thermophiles showing an absolute methionine requirement would appear to be more advantageous for methionine assay, as the media need not be sterile when incubated at 55° C or higher.

Transmethylations in bacteria have been described (11, 20). Methionine can be synthesized from homocystine plus a methyl donor, with B_{12} serving as a methyl activator (13, 19). That B_{12} intervenes in the synthesis of methionine is borne out in experiments by Davis and Mingioli (12). They showed that certain mutants requiring vitamin B_{12} accumulated a compound which in synthrophic experiments feeds another mutant responding to homocystine as well as methionine. The accumulated compound was claimed to be homocystine. Huennekens and Osborn (14) have reviewed the evidence for the role of B_{12} in methyl synthesis.

A survey of the nutritional requirements of mesophiles and thermophiles over a wide temperature range would no doubt reveal evidence of alternate pathways for syntheses, as some metabolic pathways become inactivated by thermal denaturation of key enzymes. It should be mentioned that for many thermophilic bacilli it is difficult to devise media which will support growth below their minimum temperature range in ordinary media, even when the minimum temperature is as high as 40° C. Preliminary experiments have shown us that attempts to lower the high minimal temperature cause extreme nutritional imbalance.

Acknowledgment

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CHROMATOGRAPHY OF COLIPHAGES T_1 AND T_2 ON COLUMNS OF CALCIUM PHOSPHATE[1]

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Abstract

Coliphages T_1 and T_2 were efficiently adsorbed from dialyzed lysates by small columns of calcium phosphate. Elution of the columns with phosphate buffers at low molarity (0.02–0.03) removed much of the non-infectious material; the phage could subsequently be eluted in more concentrated and purified form with higher buffer concentrations (0.06 *M* or greater). The chromatographic behavior of T_1 and T_2 is contrasted and the influence of non-phage constituents of the lysate upon the chromatographic separation is discussed.

Introduction

High-speed centrifugation is the method most frequently used for the concentration of bacteriophages; alternate cycles of low- and high-speed centrifugation can effect the partial purification of bacteriophage preparations (1). The centrifugal method is not entirely satisfactory, however, and it has been recognized (5) that other techniques, utilizing different principles, should be applied, perhaps as adjuncts to centrifugation, when phage preparations of a high degree of purity are required. In 1953, Putnam (5) reviewed the literature dealing with reversible adsorption as a means of purifying bacteriophage and expressed the hope that improvements in chromatographic methods might lead to development of a rapid and simple method for phage purification. Since then there have been a number of reports (9, 11) on the chromatography of bacteriophages and, in addition, chromatographic methods have been widely applied to the purification and concentration of animal (4,7,8) and plant (2) viruses.

Tiselius (10) reported that columns of calcium phosphate were of particular value in the purification and concentration of proteins because of the affinity of this substance for large molecules, and its failure to adsorb smaller molecules strongly. Taverne *et al.* (7) achieved excellent results in the purification of influenza virus using calcium phosphate columns. The present authors, lacking centrifugal equipment, undertook to apply chromatographic techniques using calcium phosphate in the hope of obtaining concentrated and partially purified phage preparations for serological studies.

Methods

Crude lysates of *E. coli* B phages T_1 and T_2 were prepared in Difco nutrient broth supplemented with 5 g NaCl per liter. In preliminary batch trials with calcium phosphate and crude lysates of the phages it was found that no adsorption of the phage occurred unless the lysate was first dialyzed,[4] in

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[4]All dialyses were carried out against several changes of distilled water.

which case the adsorption level was extremely high. Dialysis resulted in a marked increase in plaque-forming units (P.F.U.) in the lysate probably due to the phenomenon reported by Sagik (6). After dialysis, phage titers in excess of 10^{10} P.F.U. per ml were routinely obtained.

Calcium phosphate in the brushite form was prepared by the method of Tiselius *et al.* (10) which consisted, briefly, of mixing, dropwise and with constant agitation, equal volumes of 0.5 *M* $CaCl_2$ and 0.5 *M* Na_2HPO_4. The precipitate so formed was washed several times with water and stored in 0.001 *M* phosphate buffer, pH 6.8. Columns were prepared in a glass tube of 1 cm I.D. fitted with a stopcock and using about 0.5 g calcium phosphate (dry weight). The columns were supported with cotton wool and were covered with a layer of the same material. After light tapping of the tube to pack the column, the adsorbent was washed with about 20 ml of glass-distilled water. Columns prepared in this way were 15 mm in height and had a retention volume of about 2 milliliters. Flow rates were in the order of 0.5–1.0 ml per minute with a 20-cm fluid head.

After adsorption of phage on the columns stepwise elution was carried out with phosphate buffers (pH 6.8) of increasing molarities. Fractions were collected in 2-ml quantities, usually seven such fractions for each buffer strength used. Phage titrations were made on the fractions using the double layer plating technique and nutrient agar supplemented with NaCl. *E. coli* B served as the plating host. Estimates of the relative quantity of protein in the fractions were made colorimetrically using Folin-Ciocalteau reagent as described by Kabat and Mayer (3). Protein in the fractions was expressed relative to a standard solution of crystalline bovine serum albumin (Nutritional Biochemicals).

For electron microscopy, the fractions were dialyzed against distilled water and mounted directly or sprayed on formvar-covered grids. The preparations were shadowed with palladium, received a protective coating of carbon, and were examined in a Philips EM 75 B electron microscope.

Results

To measure the capacity of a column of calcium phosphate a fresh T_2 lysate having a plaque count after dialysis of about 2×10^{10} P.F.U. per ml was prepared and 200 ml of this lysate was passed through a column. Successive samples of the effluent were collected and plaque counts of these indicated that adsorption of the phage from the first 100 ml was greater than 99.9%. Aliquots taken after the first 100 ml of effluent showed titers gradually approaching that of the original lysate, indicating that the column had reached capacity at about 100 ml or at a phage input of 2×10^{12} P.F.U. The effluent was paler in color than the dialyzed lysate and a very dark band of color on the column confirmed that much of the pigmented material in the lysate had been retained by the adsorbent.

Elution trials were carried out using considerably less phage than the maximum just indicated. In a typical experiment 10 ml of a dialyzed T_2 lysate

having a titer of 3×10^{10} P.F.U. per ml were passed through a column. As shown in Fig. 1 elution with 0.03 *M* phosphate buffer yielded about 12% of the input infectivity while subsequent elution with 0.2 *M* buffer yielded about 68% of the input. The 0.03 *M* eluate was very dark in color while the 0.2 *M* eluate was very pale. In the tube containing the most P.F.U. there was marked opalescence.

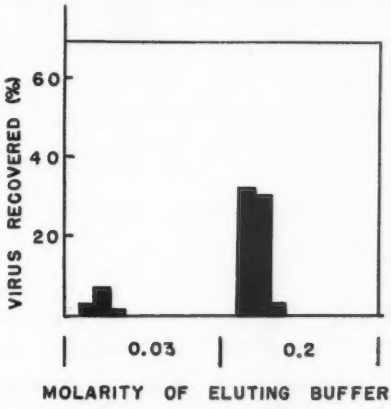


FIG. 1. Elution of coliphage T_2 from a calcium phosphate column. Input = 10 ml dialyzed lysate containing 3×10^{10} P.F.U. per ml.

Serologically the two "fractions" separated in this way appeared to be identical on the basis of infectivity neutralization by anti-T_2 serum and it seemed likely that the separation on the column was a chromatographic artifact. Further evidence of this was obtained when the two fractions were dialyzed and rechromatographed. Figure 2A shows the result of rechromatographing the small, highly colored fraction eluted from an earlier column with 0.05 *M* buffer. Once again elution revealed evidence of fractionation, the low molarity buffers containing infectivity totalling about 1% of the input, and a major fraction in 0.2 *M* buffer. The total yield was extremely low, about 35% of the input. In Fig. 2B are shown the results of rechromatographing the major fraction from an earlier column. In this case elution with buffers of strengths below 0.05 *M* yielded no phage while elution with 0.2 *M* buffer yielded a total of about 80% of the input.

The chromatographic homogeneity of the "purified" preparation (Fig. 2B) suggested that the presence of non-viral material might have been responsible for the heterogeneity observed in chromatograms of crude lysate. To test this possibility, two columns were prepared, one of which was pretreated by passing through it 50 ml of dialyzed nutrient broth. Both columns then received 5 ml of a T_2 preparation previously concentrated by elution from a calcium phosphate column with 0.2 *M* buffer (comparable to the chromatographically homogeneous fraction described above). The columns were then eluted first with 0.03 *M* buffer and then with 0.2 *M* buffer. The results are shown in Fig. 3.

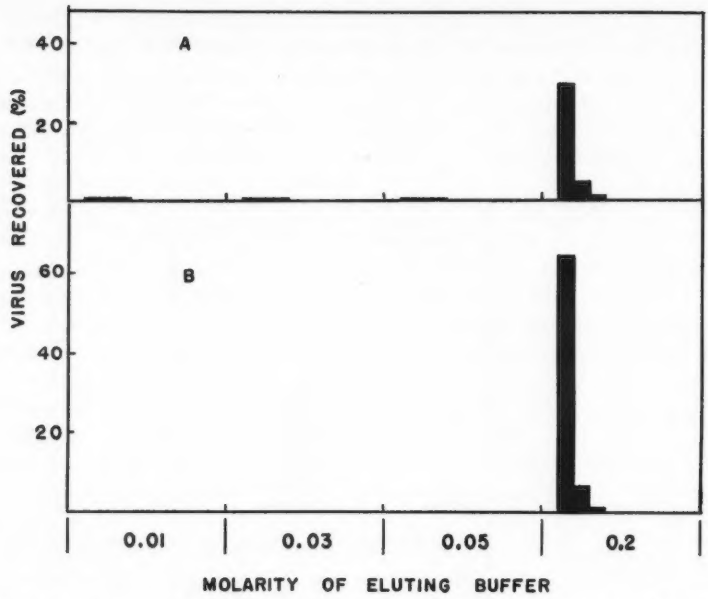


FIG. 2. Rechromatography of the two "fractions" from Fig. 1; (A) 0.03 *M* fraction, (B) 0.2 *M* fraction.

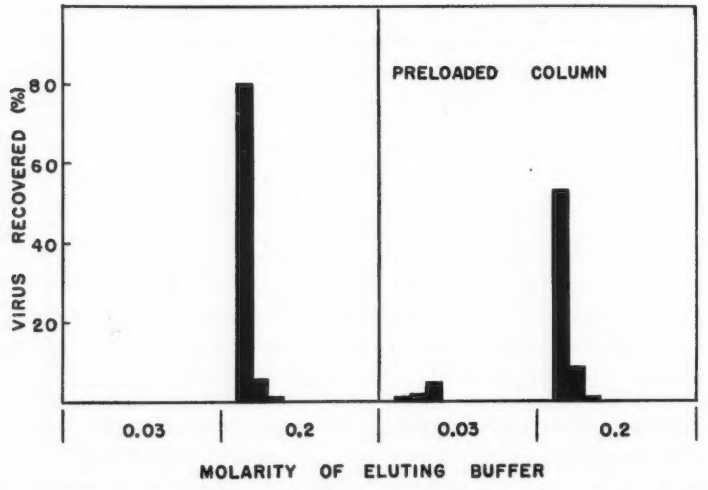


FIG. 3. Influence of medium constituents on elution of coliphage T_2 from calcium phosphate columns. Column on right received 50 ml dialyzed nutrient broth before "purified" T_2 was added.

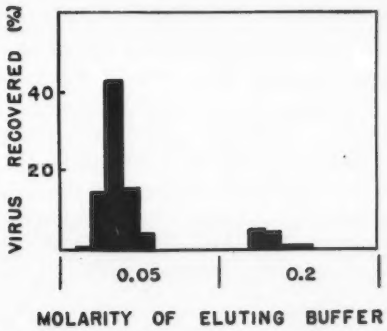
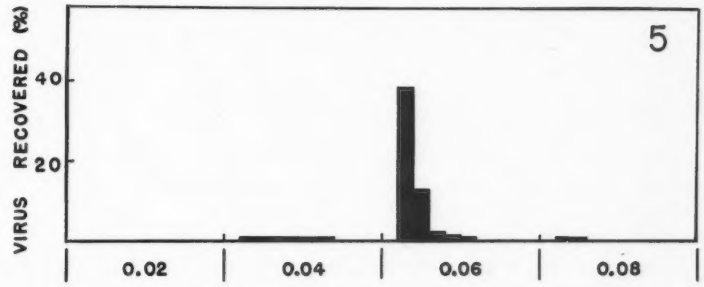


FIG. 4. Elution of coliphage T_2 from an overloaded calcium phosphate column. Input = 200 ml dialyzed lysate containing 2×10^{10} P.F.U. per ml.



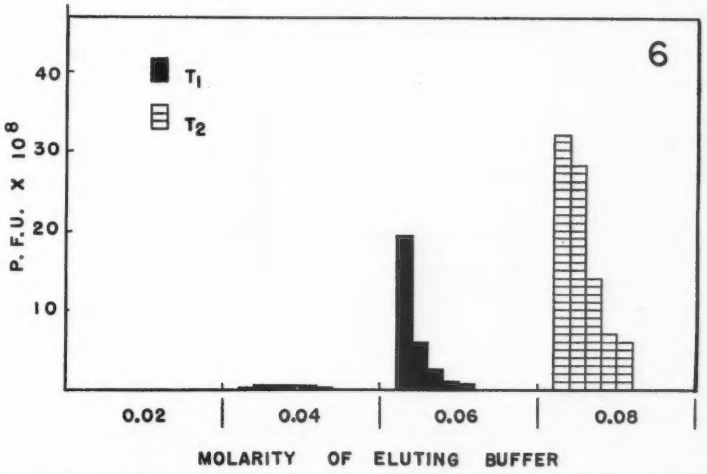


FIG. 5. Elution of coliphage T_1 from a calcium phosphate column. Input = 5 ml dialyzed lysate containing 6×10^{10} P.F.U. per ml.

FIG. 6. Separation of coliphages T_1 and T_2 by stepwise elution from a calcium phosphate column to which a mixture of the phages had been added.

The untreated column, as expected, yielded phage only in 0.2 *M* buffer, the 0.03 *M* eluate was free of infectivity. Elution of the pretreated column with 0.03 *M* buffer, however, yielded about 8% of the input P.F.U. The total yield from the pretreated column was only 72% compared with 87% from the untreated column. Further evidence of the interfering effect of non-viral material in the lysate was obtained when 200 ml of dialyzed T_2 lysate were passed through a column, a quantity well beyond the column capacity. Of the phage adsorbed, 74% was subsequently eluted with 0.05 *M* buffer, as shown in Fig. 4, while only 10% was eluted in 0.2 *M* buffer.

Further experiments designed to determine the minimum buffer concentration required to elute T_2 from calcium phosphate columns showed that the major portion was removed in 0.08 *M* phosphate buffer, pH 6.8; however, at this buffer concentration there was considerably more tailing than was apparent with 0.2 *M* buffer. This result was not unexpected in view of the observations made by Matheka and Armbruster (4) on the elution of influenza virus from an ion exchange resin.

Adsorption of dialyzed T_1 lysate on a calcium phosphate column and subsequent elution resulted in the pattern shown in Fig. 5. A small fraction of the input (about 5%) was eluted in 0.04 *M* buffer and about 55% in 0.06 *M* buffer. Rechromatography of the major fraction yielded very similar results and, so far, the marked change in elution properties with column passage observed with T_2 has not been obtained with T_1. To learn whether the two phages behaved independently, a mixture of T_1 and T_2 (each previously chromatographed) was adsorbed to a column and eluted with a range of buffers. The results, presented in Fig. 6, showed that coliphages T_1 and T_2 could be separated on the basis of their respective affinities for calcium phosphate.

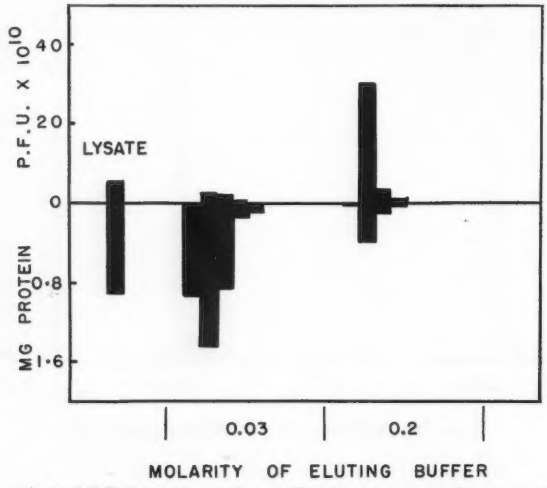


FIG. 7. Protein and P.F.U. in eluates from a T_2 chromatogram. Input = 20 ml dialyzed lysate containing 6.3×10^{10} P.F.U. per ml.

PLATE I

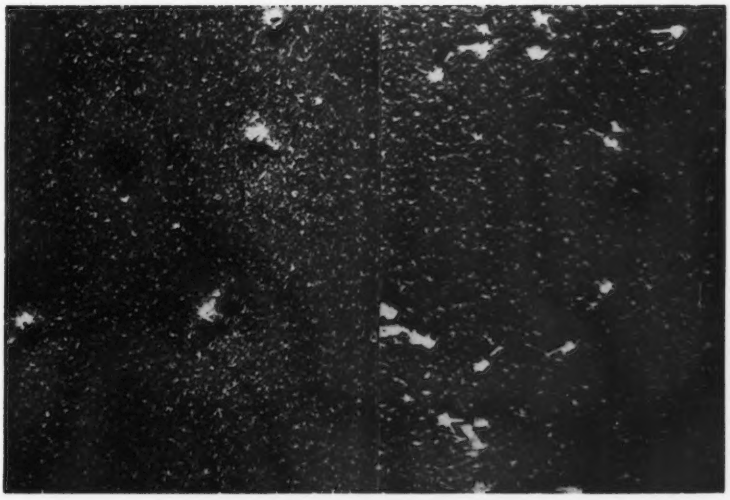


FIG. 8. Electron micrographs of a dialyzed T_2 lysate (left) and the 0.2 *M* fraction (right) after two cycles of adsorption to and elution from calcium phosphate columns.

Mention has already been made of the removal of colored compounds from T_2 suspensions by the technique outlined here. To obtain further evidence of the degree of purification achieved, a T_2 lysate was subjected to column passage and elution with 0.03 *M* and 0.2 *M* phosphate buffer. Protein determinations and plaque counts were made on fractions of the eluates. The results are shown in Fig. 7. Protein was concentrated in the 0.03 *M* eluate while the infectivity was concentrated in the 0.2 *M* eluate. These data suggested that considerable purification had been achieved. Electron micrographs of T_2 preparations "purified" by two cycles of adsorption to and elution from calcium phosphate still showed, however, many small electron dense particles (Fig. 8). The nature of these particles, presumably of host origin, has not been established.

Discussion

Chromatography on calcium phosphate columns provides a simple means of concentrating the two phages studied; concentration by a factor of 10× could be readily achieved. The data also indicate that the chromatographic method may be useful in the purification of phage preparations; however, the electron micrographs of T_2 "purified" by this means suggest, by reason of the size difference between the phage and accompanying particles, that the method would be more useful in conjunction with centrifugation.

The phages studied are adsorbed much less strongly by calcium phosphate than is either influenza virus (7) or herpes virus (8) as reported by Taverne and her associates. We have made preliminary trials with an actinophage which show it to be even less strongly adsorbed than the coliphages. It would be interesting if this low affinity for calcium phosphate, relative to that shown by animal viruses, proved to be a general property of microbial phages. Should this be the case, the method reported here may be of limited value in phage purification since, with very weakly held phages, the buffer range over which non-viral material could be eluted before virus appeared in the eluate would be too restricted for effective separation.

The elution pattern of T_1 seems analogous to that obtained for a protein with an extended elution range by Tiselius *et al.* (10), who interpret this pattern as indicating a markedly curved adsorption isotherm. In contrast, T_2, with the same stepwise changes in eluant molarities showed a sudden increase in R_f to a value approaching 1. This behavior has also been shown (10) to be characteristic of certain proteins.

Data have been presented to show the contribution of non-phage constituents of the lysate to the elution pattern obtained with T_2. Indeed, in excessively loaded columns the non-phage materials (though only weakly adsorbed) may carry some phage along with them during elution. Tiselius *et al.* (10) have pointed out that this "displacement effect" can give rise to apparent chromatographic heterogeneity.

Acknowledgment

The authors gratefully acknowledge the technical assistance of Miss C. Mock.

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HEXOKINASE ACTIVITY OF YEASTS GROWN ON BIOTIN-RICH AND BIOTIN-DEFICIENT MEDIA[1]

ROLF SIEPMANN[2] AND JAMES C. MACDONALD

Abstract

Two strains of *Saccharomyces cerevisiae* and one of *Candida parapsilosis* were grown on biotin-deficient and on biotin-rich media and the hexokinase activity in cell-free extracts determined. Extracts from *C. parapsilosis* grown on the biotin-deficient medium had more hexokinase activity than extracts from the organism grown on biotin-rich medium. The hexokinase activity of extracts from these yeasts grown on biotin-deficient medium was not increased by biotin, or inhibited by avidin dissolved in saline. The results do not confirm the possibility of a direct role for biotin in hexokinase activity.

Introduction

Winzler *et al.* (14) and Moat and Lichstein (6) have shown that oxidation and fermentation of glucose by a biotin-deficient yeast (*Saccharomyces cerevisiae* 139) was, under certain conditions, increased by the addition of biotin. Williams *et al.* (13) suggested that hexokinase was one of the enzymes affected by biotin deficiency, after they found that extracts of *S. cerevisiae* 139, grown on a biotin-deficient medium, contained less hexokinase than extracts from cells grown on a biotin-rich medium. However, the addition of biotin to these extracts had no effect on the activity. Strauss and Moat (11) prepared extracts of the same organism grown on biotin-deficient medium, but obtained a stimulation of hexokinase activity of the extracts by adding biotin. Both their method of preparation of the extracts and their method of measurement of hexokinase activity differed from those used by Williams *et al.* (13). Williams *et al.* used sonic extracts and an assay for hexokinase based on the phosphorylation of 2-desoxyglucose, while Strauss and Moat used extracts obtained with a Nossal disintegrator, and coupled the hexokinase reaction to endogenous glucose-6-P (glucose-6-phosphate) dehydrogenase and measured TPN (triphosphopyridine nucleotide) reduction.

In the present work, an attempt was made to repeat the work of Strauss and Moat and to study other yeasts in the same manner. Cell-free preparations from three yeasts grown on biotin-deficient media were made and analyzed for hexokinase activity by the method of Strauss and Moat.

Materials and Methods

Organisms and Growth Conditions

The organisms used in the present work were *Saccharomyces cerevisiae* ATCC 9896 (Fleischmann strain 139), *S. cerevisiae* ATCC 7754, and *Candida parapsilosis* (Ashford) Langeron and Talice. All these organisms required

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biotin for growth, for no growth was obtained in the absence of biotin in the medium of Snell *et al.* (8). Biotin-free sucrose for media was prepared by the method of Winzler *et al.* (14). The various rotary shakers used for growing *S. cerevisiae* were operated at 230 r.p.m. or 115 r.p.m., 1-in. radius of motion; or for growing *C. parapsilosis*, at 278 r.p.m., radius of motion 0.5 in.

For the inoculum, the organisms were subcultured in 50 ml of sterile glucose medium (13) in 250-ml Erlenmeyer flasks on a rotary shaker for 18–24 hours at 30° C (*S. cerevisiae*) or at 25° C (*C. parapsilosis*). The cells were then washed three times with 50 ml of sterile 0.8% saline and suspended in 50 ml sterile saline to give an optical density (O.D.) of 0.06 to 0.56 (Coleman Spectrophotometer, 650 mμ, 19-mm diameter round cuvettes). The suspension was diluted to 1 part in 100,000 with sterile saline, and 1 ml used to inoculate 1 liter of growth medium (Strauss and Moat (11)) of pH 4.7–4.9; or 0.1 ml to inoculate 10 ml of medium for biotin assay (8). Growth medium was sterilized in 2-liter Erlenmeyer flasks (1 liter per flask), inoculated, and incubated on a rotary shaker, or 500 ml was sterilized in 2.5-liter Fernbach flasks, inoculated and incubated without shaking. Biotin-rich or biotin-deficient media were made by adding 2.5 μg biotin per liter or 0.020 μg per liter, respectively, to the medium of Strauss and Moat (11).

Preparation of Extracts

After the growth period, the cells were harvested by centrifuging, and equal wet weights of cells from the biotin-rich and the biotin-deficient media were washed three times with 50 ml 0.8% saline. The cells were disintegrated at 0° C using the method of Nossal (7) (10 g glass beads of .21- to .30-mm diameter, 10 ml of 0.8% saline, and 1.8–2.0 g wet weight of yeast were added to the steel capsule). The yeast was disrupted by shaking for three 30-second intervals with cooling of the capsule and contents for 3 minutes in an ice bath between the intervals. Intact cells and debris were removed by centrifuging at 20,000 $\times$ *g* for 20 minutes at 0° C. The supernatant was decanted and recentrifuged if necessary, and then diluted for immediate use in the experiments that provided the data presented in Tables II and III.

Assays

Hexokinase activity was measured by coupling with glucose-6-P dehydrogenase at 25° C and measuring TPN reduction at 340 mμ in 1-cm cuvettes with a Beckman DU spectrophotometer (11). Readings were taken at 1- or 2-minute intervals after addition of the enzymes, and plotted on graph paper to determine the maximum rate of TPN reduction. In some experiments, no glucose-6-P dehydrogenase was added, since the extracts contained this enzyme. In other experiments, 0.1 ml of a glucose-6-P dehydrogenase preparation was added, which was, at least, a 10-fold excess of the amount needed to produce maximum rates of TPN reduction when measuring hexokinase at the level present in our experiments. A commercial preparation of hexokinase (Pabst Laboratories, Milwaukee, Wisconsin) was assayed by the method of Berger *et al.* (1).

Glucose-6-P dehydrogenase was measured with the same cofactors present as for the assay of hexokinase, but with glucose-6-P as the substrate.

Avidin was prepared and assayed by the method of Eakin *et al.* (2). A commercial preparation (Nutritional Biochemical Corporation, Cleveland, Ohio) was also used in some experiments. Protein was estimated by the method of Stadtman *et al.* (9).

ATP (adenosine triphosphate), TPN, and glucose-6-P were obtained from Nutritional Biochemicals Corporation. The glucose-6-P dehydrogenase preparation was supplied by Dr. E. R. Blakley of this laboratory and was the supernatant from sonically disrupted *Leuconostoc mesenteroides* cells. The preparation contained no hexokinase, 6-phosphogluconic acid dehydrogenase, glucose, or glucose-6-P.

Results

The possibility that glucose-6-P dehydrogenase was limiting the reaction rate in the assay for hexokinase in the work of Strauss and Moat (11) was tested as shown in Table I. An enzyme mixture of dialyzed commercial hexokinase and glucose-6-P dehydrogenase was made so that the results obtained by Strauss and Moat (see Table III in reference 11) with a cell-free extract of yeast, and glucose as the substrate, were duplicated (Table I) using 0.3 ml of the mixture. The results that Strauss and Moat obtained with a 1:5 dilution of the extract, and glucose-6-P as the substrate, were also duplicated by 0.3 ml of a 1:5 dilution of our mixture. With glucose as the substrate, the rate of TPN reduction by 0.3 ml of our mixture was markedly stimulated by the addition of more glucose-6-P dehydrogenase, indicating that this enzyme could also have been limiting in the yeast extracts of Strauss and Moat. However, the rate was also increased by the addition of more hexokinase, which showed that with such a mixture, increase in the amount of both enzymes stimulated the rate of TPN reduction.

TABLE I

Measurement of TPN reduction in an enzyme mixture under various conditions

Enzyme	Substrate	O.D. × 10^3 at 340 m μ*			Maximum rate, $\frac{\Delta O.D. \times 10^3}{\text{minute}}$
		4 min	8 min	12 min	
0.3 ml mixture	Glucose	11 (11)	36 (33)	69 (61)	8.3
Mixture, diluted 1/5, 0.3 ml	Glucose-6-P	36 (37)	76 (78)	113 (112)	9.4
0.3 ml mixture + 0.1 ml glucose-6-P dehydrogenase	Glucose	78	138		19.4
0.3 ml mixture + hexokinase†	Glucose	21	56	99	10.5
1.0 ml mixture	Glucose	73	177		25.4
0.3 ml mixture + 0.1 ml glucose-6-P dehydrogenase	None	0	0	0	

*The figures in parentheses represent the results obtained by Strauss and Moat (see Table III in reference 11) for an extract of biotin-deficient yeast.

†The amount of hexokinase added was equal to that already present in 0.3 ml mixture.

NOTE: Each cuvette contained 5 μmoles of substrate, 0.2 mg of TPN, 0.2 mg of ATP, 1.0 ml of 0.1 *M* phosphate buffer at pH 6.5, and 1.0 mg of $MgSO_4.7H_2O$. Final volume 3.0 ml. The zero time reading was taken within 30 seconds after addition of the enzymes.

TABLE II

Growth conditions for yeasts, cell and extract yields, and enzyme activities of the cell-free extracts*

Organism	Experiment	Growth conditions	Biotin in medium μg/liter	Yield of cells (g wet-weight /liter)	Mg protein /ml cell extract	Specific activity† with: No substrate	Glucose	Glucose + glucose-6-P dehydrogenase	Glucose-6-P
Saccharomyces cerevisiae ATCC 9896	1	94 hours shaking at 30° C	0.020	0.8	1.8	0.006	0.015	0.069	0.030
			2.5	16.6	12	0.048	0.10	0.60	0.15
	2	141 hours stationary at 30° C	0.020	1.2	0.76	0	0.056	0.48	
			2.5	9.0	11	0.003	0.11	0.72	0.14
Saccharomyces cerevisiae ATCC 7754	3	46 hours shaking at 30° C	0.020	1.0	12	0.006	0.087	0.35	0.15
	4	46 hours shaking at 30° C	0.020	1.0	12	0.003	0.090	0.63	(0.20)
			2.5	6.1	14	0	0.10	0.57	(0.16)
	5	166 hours shaking at 30° C	0.020	2.2	4.1	0.046	0.074	0.20	0.17
			2.5	21	14	0.024	0.080	0.37	0.13
Candida parapsilosis	6	67 hours shaking at 25° C	0.020	4.4	4.5	0	0.50	1.2 (0.74)	(1.2)
			2.5	22	4.8	0	0.24	(0.33)	(0.43)
	7	71 hours shaking at 25° C	0.020	5.6	3.8	—	0.47	1.0	1.7
			2.5	29	5.3	—	0.19	0.49	0.43
	8	114 hours shaking at 25° C	0.020	9.0	6.9	(0.02)	0.54	1.1	1.5
			2.5	33	6.3	(0)	0.16	0.44	0.45

*Fresh cell-free extracts were used for most experiments. The figures in parentheses were obtained with extracts stored up to 7 days at −30° C.
†Specific activity = change of optical density per minute per milligram of protein. Each cuvette contained 5 μmoles of substrate, 0.6 mg of TPN, 1.0 mg of ATP, 1.0 ml of 0.1 *M* phosphate buffer at pH 6.5, and 1.0 mg of $MgSO_4.7H_2O$. Final volume 3 ml.

With 0.3 ml of the mixture and glucose as the substrate, there was a lag of about 8 minutes before the maximum rate of TPN reduction was obtained, but with 1.0 ml of the mixture the lag was only 3 minutes (Table I). This increase of the amount of the enzyme mixture by 3.33 times caused an increase in the maximum rate of TPN reduction, as calculated graphically, of 3.06 times, but an increase in the amount of TPN reduction in the first 4 minutes of 6.04 times. Therefore the maximum rate of TPN reduction in such a mixture was a better measure of the relative amount of enzyme used than was the rate over the initial period. With 0.3 ml mixture and 0.1 ml glucose-6-P dehydrogenase, the maximum rate of TPN reduction with glucose was obtained at the start of the reaction without any lag, and this was also the case when glucose-6-P was the substrate.

Table II shows the growth conditions for the yeasts used and the enzyme activities of the extracts. With the medium of Strauss and Moat, the amount of biotin in the biotin-deficient medium limited the amount of growth, as determined by wet weight, to about one-fourth (or less) of that obtained using the biotin-rich medium (Table II). Because of the very small inoculum used, the growth periods necessary to obtain adequate yields of yeast cells were longer than those used by Strauss and Moat (11). The small inoculum was used to minimize the possibility of adding traces of biotin with the inoculum. The experiments of Strauss and Moat were done with yeast grown in stationary culture (private communication), so *S. cerevisiae* ATCC 9896 (Fleischmann strain 139) was grown in this manner in experiment 2.

The analyses for hexokinase activity and glucose-6-P dehydrogenase activity were done by one of the methods used by Strauss and Moat (see Table I in reference 11), except that the concentration of TPN was increased. Preliminary tests with each extract were made to find the dilution of extract necessary to obtain a convenient rate of TPN reduction. With glucose as the substrate, a lag was observed before the maximum rate of TPN reduction was obtained, much the same as was observed with the enzyme mixture used to obtain the results shown in Table I (see above). Because of this, the specific activity was calculated on the basis of maximum rate of TPN reduction.

The validity of expressing the results as specific activity (optical density change per minute per milligram of protein) was tested with some of the extracts used in the experiments in Table II. It was not possible to do this with fresh extracts because of the time needed to complete the experiments listed in Tables II and III. However, the extracts, stored at $-30°$ C, retained over half of their original activity and the specific activity with glucose, glucose plus glucose-6-P dehydrogenase, and with glucose-6-P, was approximately constant ($\pm 10\%$) over a three- to five-fold range of dilution using the assay conditions of Table II. The endogenous specific activity (no substrate added), however, was not constant but fell rapidly with dilution. For this reason, the endogenous specific activities in Table II are probably a maximum figure, for they were obtained with the highest concentration of enzyme used in the particular experiment.

TABLE III

Enzyme activity of cell-free extracts from biotin-deficient yeasts

Organism	Experiment*	Specific activity† with glucose as substrate					
			+ biotin	+ avidin	+ glucose-6-P dehydrogenase	+glucose-6-P dehydrogenase + biotin	+ glucose-6-P dehydrogenase + avidin
Saccharomyces cerevisiae ATCC 9896	1	0.015	0.015	0.015	0.069	0.072	0.070
	2	0.056	0.057	0.045	0.48	0.46	0.42
Saccharomyces cerevisiae ATCC 7754	3	0.087	0.083	0.081	0.35	0.37	0.32
	5	0.074	0.072	0.073	0.20	0.20	0.19
Candida parapsilosis	6	0.46	0.48	0.41	1.2	1.2	1.0
	7	0.47	0.48	0.38	0.94	0.92	0.77
	8	0.53	0.54	0.46			

*See Table II for conditions of growth in these experiments.
†Conditions as in Table II. Biotin, 12.5 μg per cuvette. Avidin, 0.025 units in 0.2 ml of 1% $(NH_4)_2SO_4$ in experiments 2, 3, 6, 7, and 8, and 0.10 units in 0.1 ml of 0.8% NaCl in experiments 1 and 5.

Even though all the cell-free extracts used in Table II had glucose-6-P dehydrogenase activity, the activity with glucose as the substrate was increased by the addition of more glucose-6-P dehydrogenase, indicating that the latter enzyme was not present in sufficient concentration for maximum rates in the assay for hexokinase. The activity of hexokinase in *C. parapsilosis* extracts, as measured either with or without added glucose-6-P dehydrogenase, was higher when the organism was grown on biotin-deficient medium than when it was grown on biotin-rich medium, and similar results were obtained for the activity of glucose-6-P dehydrogenase. The hexokinase activity in extracts of *S. cerevisiae* seemed to be either similar (experiment 4) or lower (experiments 1, 2, and 5) when the organism was grown on biotin-deficient as compared to biotin-rich medium, although this was not shown with much certainty because of high endogenous activity in experiments 1 and 5.

Although the extracts were obtained by disintegrating the same wet weight of cells, in some of the experiments (Table II) there was a higher concentration of protein in the extracts from biotin-rich as compared to biotin-deficient cells. In experiment 1, examination with the microscope showed that a large number of lysed cells were present in the biotin-deficient medium at the time of harvest. The lower yield of protein from biotin-deficient cells appeared to be paralleled by a lower hexokinase specific activity (as measured with added glucose-6-P dehydrogenase), but the reason for this was not investigated further.

Freshly prepared extracts from cells grown on biotin-deficient medium used in Table II were also used in the work outlined in Table III. Cuvettes containing avidin or biotin were run with the same concentration of extract as the controls. Hexokinase activity was measured with or without the addition of glucose-6-P dehydrogenase, and biotin gave no stimulation of activity under either of these conditions even though the amount of biotin was four times that used by Strauss and Moat (11). Avidin decreased the activity slightly when dissolved in ammonium sulphate solution, but not when dissolved in sodium chloride solution. This effect was further investigated with the extract from experiment 7, and a larger volume of the avidin solution was tested

(Table IV). The data in Table IV show that the inhibition of the activity was due to the ammonium sulphate in the avidin solutions and not to the avidin. A similar inhibition could be obtained with sodium sulphate, but not with ammonium chloride, so the inhibition appears to be caused by sulphate ions.

TABLE IV

Effect of avidin solutions on the enzyme activity of a cell-free extract of *Candida parapsilosis**

Specific activity† with glucose as substrate				
	+ $(NH_4)_2SO_4$	+ $(NH_4)_2SO_4$ + avidin	+ NaCl	+ NaCl + avidin
0.38	0.25	0.24		
0.42			0.37	0.42

*This extract was obtained in experiment 7 from *Candida parapsilosis* grown on biotin-deficient medium and was stored in the deep freeze before use.
†Conditions as in Table II; 0.6 ml of 1% $(NH_4)_2SO_4$ with or without 0.075 units of avidin, or 0.75 ml of 0.8% NaCl with or without 0.75 units of avidin, was added per cuvette as indicated.

The amount of avidin used in the above experiments was found by assay to be sufficient to bind traces of biotin in the enzyme preparations. No detectable amount of biotin was found in the glucose and cofactors used in the hexokinase assay.

The activity of a commercial preparation of hexokinase was also not inhibited by avidin. The enzyme activity was measured by a manometric method and 2.4 units (1) of hexokinase (in 0.188 mg of the preparation) was not inhibited by 2.5 units (2) of avidin.

Discussion

In the present work an attempt was made to use the methods of Strauss and Moat (11), and to test them on yeasts more biotin-deficient than that used by these workers. Accordingly, the biotin concentration of their growth medium was lowered from 0.025 μg/liter to 0.020 μg/liter for our experiments, and very dilute inoculum used to minimize the transfer of biotin in the inoculum. Strauss and Moat were able to obtain satisfactory yields of *S. cerevisiae* (Fleischmann strain 139) after growth for 18 hours at 32° C in stationary culture, while with our inoculum and the same organism, adequate growth in stationary culture was obtained only after 141 hours at 30° C, and no visible growth at all in 18 hours.

The evidence that Strauss and Moat present for the stimulation of hexokinase activity by biotin is based in part on studies on the influence of biotin on fermentation by air-dried cells and in part on a more direct measurement of hexokinase by photometric method (11). They found that biotin had to be preincubated with the dried cells before a stimulation of fermentation could be obtained, while biotin immediately stimulated the hexokinase activity of cell-free extracts as measured by the photometric method. The latter assay requires that glucose-6-P dehydrogenase be present in excess and is therefore much simpler than an assay based on fermentation, which requires that 11

enzymes be present (3). These factors, and the fact that in preliminary experiments we prepared dried cells that had hexokinase activity but did not ferment, led us to use cell-free extracts and the photometric assay. Kiesow, who used a similar assay to measure hexokinase in frozen yeast (4), added glucose-6-P dehydrogenase to make sure that an excess was present. Strauss and Moat found that the activity of glucose-6-P dehydrogenase in their extracts was relatively high and assumed that it was non-limiting. The present work suggests that both hexokinase and glucose-6-P dehydrogenase concentrations could have limited the reaction rate in their experiments, and it is not possible to decide whether the biotin stimulation they obtained would have been more or less if excess glucose-6-P dehydrogenase had been added. However, in our experiments, no stimulation of hexokinase activity was obtained with biotin under the conditions used by Strauss and Moat, even though 12.5 μg was added per cuvette as compared to 3 μg used by these workers (11). This was also true when glucose-6-P dehydrogenase was added to the assay medium to prevent this enzyme from limiting the rate of reaction in the hexokinase assay.

Williams *et al.* found that hexokinase activity was lowered in *S. cerevisiae* 139 when the organism was grown in biotin-deficient as compared to biotin-rich media. The present work indicates that biotin deficiency does not necessarily lead to lowered hexokinase activity, for the hexokinase activity in *C. parapsilosis* was higher when the organism was grown on biotin-deficient medium than on biotin-rich medium.

Recent work by Lynen *et al.* (5), Swick and Wood (12), and Stadtman *et al.* (10) has demonstrated that biotin is involved in certain enzymatic carboxylation reactions and has provided evidence as to its mode of action. These workers did not have to use biotin-deficient media to grow the organisms from which the enzymes were isolated and yet marked inhibition was obtained with avidin. No such marked inhibition by avidin was obtained with hexokinase in the present work. Because of this and the other results presented in this paper, the authors believe that more evidence must be presented in favor of a role for biotin as a functional part of hexokinase before it is considered proved.

Acknowledgments

We are grateful to Dr. E. R. Blakley of this laboratory for supplying glucose-6-P dehydrogenase, and to Dr. N. J. W. Kreger-van Rij, Centraalbureau voor Schimmelcultures, Delft, Holland, for the identification of *Candida parapsilosis*.

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CONTINUOUS-FLOW DILUTION OF SOIL SUSPENSIONS FOR MICROBIAL ASSAY[1]

J. D. MENZIES[2]

Abstract

The principle of logarithmic dilution resulting from passage of a diluent through a solution in a closed system is used in an apparatus for obtaining samples of soil suspension of known dilution for microbial assay. The device consists of a 14-ml glass chamber containing a magnetic agitator. As sterile water flows through the chamber in which a soil sample has been placed, the dilution of the sample in the effluent depends upon the amount of water used. Samples of effluent can thus be collected for replicated plating at any desired dilution, or aliquots can be plated directly at dilution intervals selected so that some portion of the series will yield plates with the optimum number of colonies for counting. The method is simple and timesaving and should compare favorably in accuracy with conventional procedures.

Introduction

In making assays of microbial populations in soil samples by the plate-count method, it is customary to prepare soil suspensions at several preselected dilutions from which samples are drawn for plating. These dilutions are selected somewhat arbitrarily so at least one of them will yield plates with numbers of colonies in a suitable range for counting. During the diluting and plating it is difficult to maintain a uniform suspension of the soil. This is one of the reasons why plate counts from soil are extremely variable, requiring numerous replications to obtain reliable counts.

Another source of error in the standard 10-fold dilution procedure is that estimates of microbial populations in a sample based on plate counts tend to be progressively higher as higher dilutions are used. This is due to inhibitory effects between colonies that become more pronounced in crowded plates. For highest accuracy in comparative counts one should select dilutions of each sample for counting that give close to the same number of colonies per plate. Such selecting is seldom available with the few dilutions that are usually plated.

In an effort to eliminate some of these sources of variability, a continuous-flow dilution procedure has been devised that appears to be sufficiently promising to warrant further trial.

Methods

The dilution procedure is similar to that used for varying the concentration of eluent in gradient elution chromatography (1), and in providing a continuously changing concentration of pesticide in the logarithmic plot sprayer (2).

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Contribution from the Soil and Water Conservation Research Division, Agricultural Research Service, U.S. Department of Agriculture, Irrigation Experiment Station, Prosser, Washington, cooperating with the Washington Agricultural Experiment Station.
[2]Western Soil and Water Management Research Branch, Soil and Water Conservation Research Division, Agricultural Research Service, U.S.D.A., Prosser, Washington.

The principle is based on the fact that if a closed container is completely filled with a solution or suspension that is kept mixed by thorough agitation, and a diluent slowly introduced through an inflow tube with equal displacement of the mixture from an outflow tube, the concentration of the solution will decrease logarithmically with volume passed through. This may be expressed by the equation:

$$[1] \qquad C = C_0 e^{-x/v}$$

where C_0 is the original concentration, v is the container volume, x is the volume of diluent passed through, and e is 2.718. The concentration of a sample of effluent C_s may then be obtained by integrating the above equation between the desired values of x, leading to a general equation as follows:

$$[2] \qquad C_s = \frac{C_0 v(e^{-x/v} - e^{-(x+z)/v})}{z}$$

where z is the volume of the effluent sample drawn after volume x of diluent has been used.

For estimating microbial populations by plate counting, it is convenient to express the concentration of the plated sample in terms of a dilution factor by which the plate count can be multiplied. This factor can be obtained for samples of effluent from such a system by the equation:

$$[3] \qquad \text{dilution factor } (DF) = \frac{z}{e^{-x/v}(1 - e^{-z/v})}.$$

If samples of equal volume of effluent are taken at uniform intervals, the dilution factors of consecutive samples will increase progressively by a factor of $e^{y/v}$ where y is the sampling interval measured in units of volume and v is the chamber volume. From these relationships, it is easy to prepare calibration charts or tables for any particular chamber volume.[3]

The dilution apparatus, Fig. 1, utilizes a cylindrical glass chamber of about 14-ml capacity made by shortening a 50-ml glass-stoppered centrifuge tube. A small-bore inflow tube is attached on one side and a short outflow tube projects from the shoulder near the stopper. The agitator is a Teflon-covered magnetic stirring bar, slightly longer than the inside diameter of the chamber. The chamber is connected, by a short piece of tubing containing a glass bead pinch valve, to a burette in turn connected by a side arm to a reservoir of sterile water.[4] The chamber volume v is determined to a calibration mark at the base of the outflow tube, with the agitator in place.

The stopper joint and the tip of the outflow tube are the only points in the apparatus where microorganisms might be held and later dislodged at high dilutions to give an erroneous plate count. To minimize this possibility the stopper should be carefully ground in with fine carborundum and the inside

[3]Guidance and advice from R. A. Cressman and S. J. Mech on these mathematical relationships is gratefully acknowledged.

[4]Commercially available units of this apparatus include Precision Scientific Co. Mag Mix No. 65904 with Spinbar Teflon magnetic bars, 1 in. × 5/16 in. The chamber is constructed from a Corning Pyrex 50-ml centrifuge tube No. 21016B.

end polished. The stopper should be coated lightly with silicone lubricant. The tip of the outflow tube will deliver cleanly without becoming contaminated on the outside by effluent if it is smoothly bevelled and treated with a silicone water-repellent coating.

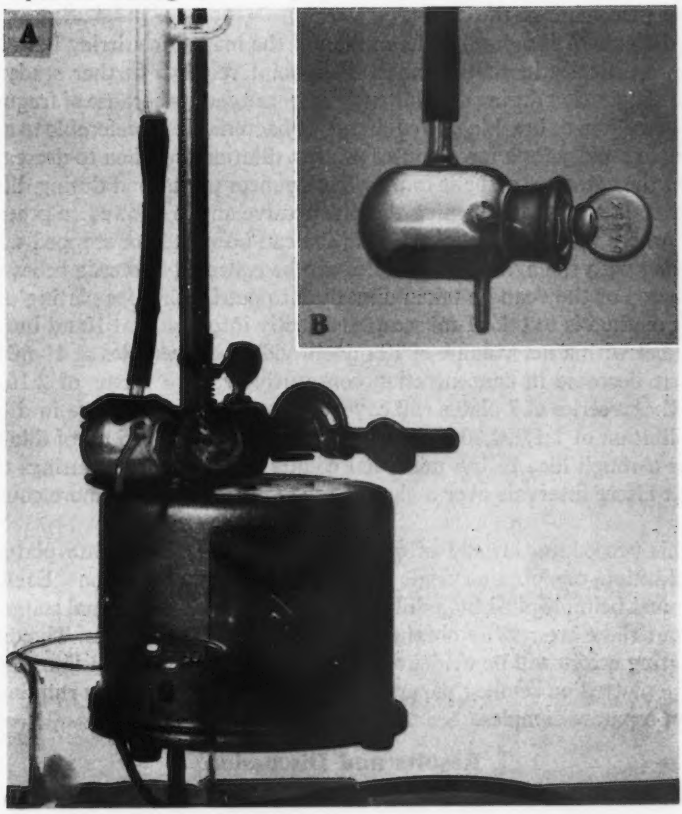

FIG. 1. Continuous-flow dilution apparatus. A. General view showing arrangement of chamber in relation to magnetic stirring motor. B. View of chamber showing location of inlet and outlet tubes.

To perform a dilution, a sample, either of soil or concentrated soil suspension, is placed in the chamber, the stopper is inserted and sterile water run in until all air is displaced through the outflow tube. The chamber is then clamped horizontally at the edge of a magnetic stirring motor, as shown in Fig. 1A. The motor speed is adjusted to give an erratic motion to the agitator. When the sample is in a satisfactory state of agitation, the pinchcock is slowly opened to fill the outflow tube. Then the water level in the burette is brought to zero and dilution can begin.

For accurate dilution of suspended particles the chamber must be placed with the outlet on the side and the rate of flow must be fast enough to draw off

the larger particles. Satisfactory results have been obtained at flow rates between 50 and 80 ml per minute. Under these circumstances tests have shown that the apparatus will quantitatively dilute soil particles up to 50 μ in size. It may be possible to extend this accuracy to include larger particles by lower placement of the outlet.

It is difficult to standardize the motion of the magnetic stirring bar since it bounces erratically in the chamber. This point requires further study since the rate of agitation during dilution may influence counts because of fragmentation of mycelium or breaking up of clumps of bacteria. It is preferable to agitate the sample rapidly for a few minutes prior to dilution and then to decrease the speed of the agitator to about four or five bounces per second during dilution.

Dilution is started by opening the pinch valve and is allowed to proceed to a point where samples are desired. Dilution can be started or stopped without interfering with the agitation. Samples can be collected in sterile tubes whenever desired or they can be taken directly into petri dishes for plating. A convenient method is to take 1-ml samples directly into dishes at 10-ml intervals. A chamber with a net volume of 13 ml will yield 1-ml samples at 10-ml intervals that decrease in concentration consecutively by a factor of 2.16. This means that a series of 7 plates will cover almost a 1000-fold range in dilution, and a dilution of 1:1,000,000 will be reached with about 165 ml of diluent. If one has a rough idea of the microbial counts in the sample, platings can be made at closer intervals over a shorter range, thus providing more countable plates.

In this procedure, instead of obtaining a number of replicate plates at a single dilution, one obtains single plates over a range of dilutions. Each plate count must be multiplied by a different factor to estimate original soil populations, but these are readily obtained from the calibration tables. The dilution and plating errors will be evident as deviations from a straight line when the data are plotted on semilog paper. Replications are obtained by running dilutions on separate samples.

Results and Discussion

The described apparatus has been tested in various modifications with a wide range of samples over a period of 6 months. It has been shown to be very accurate in diluting chemical solutions or colloidal suspensions where these have been compared with volumetric dilutions by spectrophotometric methods.

To measure the variation among plate counts from samples of particulate suspensions, several series of consecutive 1-ml samples were plated, with results shown in Fig. 2. Lines A and B show the counts obtained from duplicate dilution of a suspension of sclerotia of *Verticillium albo-atrum* plated on an alcohol–streptomycin medium. The sclerotia, which averaged 50 μ in size, were screened out from crushed dried potato stems. Lines C and D represent plate counts of total fungi from duplicate samples of soil. The slope (m) of the theoretical dilution line is depicted by E. Coefficients of variation of population estimates derived from these data were: A, 8.7%; B, 9.9%; C, 15.6%; and D, 13.7%.

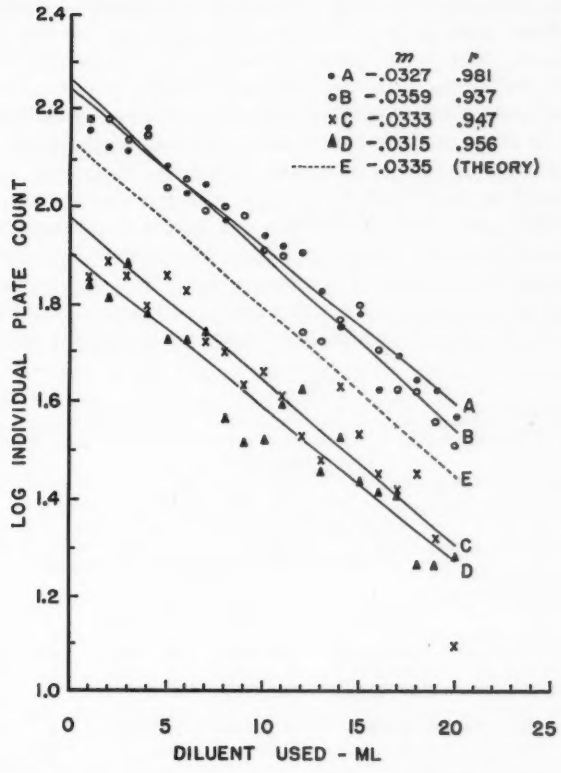

FIG. 2. Plate counts of successive 1-ml samples of a suspension of *Verticillium* sclerotia (A and B), and a previously diluted soil sample (C and D), taken during continuous-flow dilution through a 13.0-ml chamber.

Plate counts from soil samples have often given a progressively higher estimate of soil populations as the dilution is increased. The low estimates from the less dilute plates are believed to be due not only to overgrowth and masking of some colonies by others but also to prevention of growth of sensitive microorganisms by growth products from adjacent colonies. The continuous-flow dilution method automatically provides data on this effect because of the range of dilutions plated. Thus it should always be possible to select a portion of the series for counting that falls below the interference point and above the point where very low plate counts lead to excessive variability. This is illustrated by some data taken from current experiments Tables I and II.

The results in Table I indicate that interference effects may be evident in plates with fewer than 100 colonies when these are assorted soil bacteria.

Table II shows that when fungi are counted on a non-selective medium such as potato dextrose agar the error may become apparent at a concentration of about 100 fungus colonies per plate. But when bacteria are eliminated, and fungus growth restricted by the streptomycin–oxgall medium, there appears to be no serious inhibition of colony numbers until the population per plate is above 300. In all cases these counts were obtained with the aid of a wide-field binocular microscope equipped with dark-field illumination. These data are not presented as conclusive evidence on the above points, but mainly to illustrate how the continuous-flow method can be used to measure or avoid the interference error in plate counting. They do, however, suggest that the standard practice of using plate counts between 30 and 300 for microbial population estimates may be subject to considerable error when diverse types of organisms are plated together as is the case with soil suspensions.

TABLE I

Effect of dilution and homogenizing of soil suspension on plate counts and population estimates of bacteria.* Data obtained with 13.0 ml dilutor. Each determination from a single plate

		Dilution used (ml)				
		100	110	120	130	
		Dilution level				
		1:29,600	1:63,900	1:138,000	1:298,000	Av.
Homogenized†	0.5 min.	9.0 (300) ‡	10.6 (165)	10.9 (78)	13.1 (44)	10.9
"	1.0 "	8.0 (268)	12.2 (191)	13.2 (94)	13.7 (46)	11.7
"	2.0 "	9.0 (300)	10.6 (165)	10.8 (77)	12.2 (41)	10.6
"	4.0 "	9.0 (300)	9.9 (155)	9.8 (70)	14.6 (49)	10.8
"	8.0 "	7.5 (250)	9.5 (148)	11.6 (83)	14.3 (48)	10.7
	Av.	8.5	10.6	11.3	13.6	

*Millions per gram with plate count in parentheses.
†1:10 soil suspension in 1-pt container on Servall Omnimixer operated at full speed. Soil used was a fine sandy loam.
‡Plated on a soil extract – peptone – yeast extract medium.

TABLE II

Effect of media and dilution on plate counts and population estimates of fungi in sandy loam soil using continuous-flow dilutor

Diluent used (ml)	Dilution factor	Potato dextrose agar				Streptomycin–oxgall agar			
		Colonies per plate*			Estim. 1000's/g	Colonies per plate			Estim. 1000's/g
		A†	B	Av.		A	B	Av.	
50	633	302	420	361	229	518	585	551	349
55	929	260	303	281	262	410	435	422	392
60	1370	213	204	208	285	294	293	293	401
65	2010	172	153	162	326	191	241	216	434
70	2950	114	138	126	372	132	150	141	415
75	4330	109	82	95	411	111	107	109	472
80	6360	61	67	64	407	64	69	66	420
85	9340	40	51	45	420	48	53	50	467

*Each figure represents a single plate. Colonies counted with aid of a wide-field binocular microscope.
†A and B are separate samples taken from a 1:10 soil suspension. Counts represent fungous colonies only.

It is difficult to make an objective comparison of the accuracy of this dilution method with the conventional pipetting procedure. Skilled technicians can probably obtain results of similar accuracy by either method. The dilution apparatus, however, appears to eliminate many of the possible sources of human error or variability associated with the numerous manipulations required in the pipetting technique.

The dilution apparatus described can be assembled aseptically, with an adequate sterile water reservoir and appropriate air filter traps, so that it is ready for use as needed. After use the chamber can be filled with a germicide solution such as sodium hypochlorite and the apparatus left intact until another dilution series is required. The germicide can then be flushed from the chamber and a new sample introduced. The method thus eliminates the need for maintaining a supply of sterile pipettes and bottles, and tubes of sterile water. The apparatus can also be used to obtain small samples of graded concentrations of other solutions or suspensions for such purposes as dosage–response studies.

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NOTES

AEROMONAS HYDROPHILA (PSEUDOMONAS HYDROPHILA) NRC 491 AND NRC 492 ESTABLISHED AS AEROBACTER CLOACAE*

MARY T. CLEMENT AND N. E. GIBBONS

"Witness my distress,
Here's a pretty mess!"
W. S. GILBERT

Cultures NRC 491 and 492 have been used for many biochemical studies in the laboratories of the Division of Applied Biology, National Research Council, and cultures have been deposited in other collections as *Aeromonas hydrophila* or *Pseudomonas hydrophila* (NRC 491 as ATCC 11438, NCIB 8530, and NRC 492 as ATCC 11439, NCIB 8529). It was recently brought to our attention by Dr. Rudolph Hugh that these organisms do not conform to the published descriptions of *A. hydrophila* (1, 6, 7, 8). A comparison was therefore made of these cultures with two authentic strains of *A. hydrophila* (ATCC 7965, Speck strain, and McGill 6–1–A–4, a strain obtained from E. G. D. Murray).† The authentic strains have a single polar flagellum in both stained preparations and in electron micrographs, whereas NRC 491 and 492 have peritrichous flagella. The following biochemical differences were noted after incubation at 30° C for a maximum of 5 days:

	NRC 491	NRC 492	ATCC 7965	McGill 6-1-A-4
Lactose	A	A	−	−
Xylose	AG	AG	−	−
Raffinose	AG	AG	−	−
Citrate (Koser's)	+	+	−	−
Indol	−	−	+	+
Hydrogen sulphide (Kligler's)	−	−	−	+
Milk	A	A	Coagulated and digested	
Gelatin stab	−	−	Liquefied	Liquefied
Cytochrome oxidase (3)	−	−	Weak	+
Ornithine decarboxylase	+	+	−	−

The reactions of the authentic strains are typical of the species, except for the negative sulphide production by our strain of ATCC 7965. Miles and Miles (7) reported sulphide production by all authentic strains, including their culture of ATCC 7965.

Cultures 491 and 492 differ in many biochemical respects from *A. hydrophila* and exhibit characteristics of the genus *Aerobacter*. The following are typical reactions of 491 and 492 obtained by several workers: motile with peritrichous flagella; arabinose, cellobiose, dextrose, fructose, galactose,

*Issued as N.R.C. No. 5796.
†Isolated by L. Margolis (Can. Fish Culturist, **10**, 30–31 (1951)).

maltose, mannose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose, and xylose are fermented in 24 or 48 hours with production of acid and gas; fermentation of lactose with acid and gas is delayed (3–14 days) and of inositol slow (10 days) or negative; acid only produced in glycerol (7–14 days) and in rhamnose; adonitol, dulcitol, and inulin are not fermented; urease, lysine decarboxylase, and phenylalanine deaminase negative; arginine dihydrolase and orthinine decarboxylase +; IMViC − − + +; KCN and catalase +; nitrate reduced to nitrite but not gas; H_2S not produced in Kligler's medium; slow liquefaction in charcoal–gelatin (4 days). It should be noted that an anaerogenic strain of 492 was isolated from the stock culture by Dr. J. Yurack. However, the strain usually produces large amounts of gas.

These strains must be removed from the genus *Aeromonas* or *Pseudomonas* because of their peritrichous flagellation, and because of the IMViC and other biochemical reactions we would place them in the Cloaca (Aerobacter) group (2) of the genus *Aerobacter* and the species *Aerobacter cloacae* (1). Both strains have the distinguishing reactions of this species listed by Hormaeche and Edwards (5).

The interest in *Aeromonas hydrophila* in our laboratories dates from the work on 2,3-butanediol fermentation and the study by Stanier and Adams (11) of this fermentation in a number of strains of *Proteus hydrophilus* obtained from W. L. Kulp and from E. R. Hitchner. Stanier noted the active fermentation characteristics of these organisms and suggested they should be called *Aeromonas hydrophila* (10). Stanier and Adams used an unspecified strain "with particularly vigorous fermentation properties" and reported a xylose fermentation as active as that of glucose (11) although published descriptions of *P. hydrophilus* at that time (6, 8) and since (1, 7) are consistent in stating that xylose is not fermented. Dr. Hitchner supplied strains of *Proteus hydrophilus*, which he had obtained from Dr. G. B. Reed, and in a letter stated, "All of these strains have shown monotricic [sic] flagellation in our laboratory."

Several years later (1949), there was renewed interest in these organisms but freeze-dried cultures of the organisms collected by Stanier failed to grow. Replacements were requested from Dr. G. B. Reed, and two cultures were received as *Proteus hydrophilus*, the tubes labelled "salmon" and "pike". These were given the NRC numbers 491 and 492 respectively. A brief covering note stated that these two cultures were all that remained of a large collection of *P. hydrophilus* isolated in studies of 'red sore' disease of fish (8, 9). Since these organisms fermented xylose (4) and a laboratory note (single observation) indicated that they were polar flagellated, it was assumed that they were similar to the organism used by Stanier and Adams. However, a reference to citrate utilization (12) soon after these organisms were received leads us to surmise either that the original organisms were replaced by *Aerobacter cloacae* soon after receipt or that the presence of these hardier organisms may explain the survival of these two cultures in Reed's collection.

On the basis of the above, we must conclude that NRC cultures 491 and 492 are not *Proteus hydrophilus*, *Pseudomonas hydrophila*, or *Aeromonas hydrophila*, and according to the present system of classification (1) should be

designated as *Aerobacter cloacae*. Note should be made of this in the studies using cultures 491 and 492 from this Division during the period 1951 to 1959 (key references are noted below).

We wish to thank Dr. Rudolph Hugh of George Washington University, Washington, D.C., for bringing this matter to our attention and supplying his data. We are also grateful to Dr. W. H. Ewing, Communicable Disease Center, Atlanta, Ga., who has studied these strains and reached the same conclusions, and to Dr. J. Yurack, Laboratory of Hygiene, Ottawa, for assistance in checking our results.

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HYDROGEN SULPHIDE PRODUCTION BY SOME AGAROLYTIC MARINE BACTERIA[1,2]

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In studies of ecology and nutrient cycles in the sea, the anaerobic sulphate-reducing bacteria, rather than the aerobic or facultative ones, are primarily considered as prominent producers of hydrogen sulphide. During a study to be reported elsewhere, 19 cultures were isolated which resembled *Agarbacterium*, i.e. were Gram-negative, non-motile, and weakly fermentative; showed variable yellow pigmentation; and appeared to "digest" or soften agar. Identification was impeded by inadequate descriptions of the 12 *Agarbacterium* species recognized by Breed (1), of which only one produces hydrogen sulphide and six do not, and for five the reaction is not reported. Seventeen of our 19 isolates were shown to produce hydrogen sulphide. This disparity led to a study of this property by these isolates in three different media (prepared with sea water) whose compositions were as follows:

Ingredients	Broth, g/liter	SIM (Difco), g/liter	Lead acetate (Difco), g/liter
Bacto-beef extract	—	3.0	—
Bacto-peptone	—	30.0	15.0
Proteose peptone, Difco	30.0	—	5.0
Peptonized iron, Difco	—	0.2	—
Bacto-dextrose	1.0	—	1.0
Sodium thiosulphate	—	0.025	0.08
Lead acetate	—	—	0.2
Agar	—	3.0	15.0

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Over the broth medium were suspended strips of filter paper, saturated with a solution of lead acetate and then dried, a procedure found to be superior by ZoBell and Feltham (4). The other two media are solid and semisolid stab media used commonly in differentiating enteric bacteria.

After 12 days' incubation at 25° C only two cultures failed to show hydrogen sulphide production in all media, and only one in the semisolid SIM medium showed a positive test, as evidenced by a jet black color. It was found that the lead acetate stab was inadequate owing to a brown discoloration of an uninoculated control tube, and no development of a black color in any tests.

The strip method was obviously the most sensitive and therefore is the most applicable medium of the three for the detection of hydrogen sulphide production by these agarolytic marine bacteria. According to the Committee on Bacteriological Technic (2), media in which iron salts are incorporated are now generally preferred, owing to the inconvenience of the test-strip method, admitting its sensitivity and lack of inhibitory disadvantages. Clearly, any inconvenience here encountered in preparation is overshadowed by the sensitivity of the test itself and its value in aiding identification. Hunter and

Crecilius (3) stated that sufficient information is given only when both the indicator and the method itself are mentioned when a report regarding hydrogen sulphide is given. The present study emphasizes this point in reference to a group of marine bacteria lacking in complete description and suggests that a study of hydrogen sulphide production by appropriate and stated tests would be of decided importance in the identification of an isolate and the possible assessment of its contribution to the ecology.

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THE LACK OF A REVERSING EFFECT OF CERTAIN METALLIC IONS ON THE INHIBITORY ACTION OF SALICYLANILIDE[1]

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Salicylanilides exhibiting antifungal activity embody in their structure a —CONH and an ortho phenolic hydroxyl grouping. On the basis of the characteristic structural groupings present it might be assumed that salicylanilide would bind metal ions via chelation and thereby inhibit growth. Metabolic antagonism might also be considered as a possible mode of action of the salicylanilides since salicylyl-β-alanide and mandelyl-β-alanide (5) have been reported to be highly effective displacers of pantothenic acid. Our data presented here and elsewhere (2) suggest that salicylanilide and certain of its chloro-derivatives may not be acting by chelation but rather by hydrogen bonding. The key importance of the ortho hydroxyl group and the anilide group have been established.

The determination of the presence of a chelate was carried out using solubility and drop in pH effect (4). Salicylanilide, 5-chloro-salicylanilide, 5,3′-dichloro-salicylanilide and 5,4′-dichloro-salicylanilide were tested, as was glycine for comparison purposes. Each determination consisted of three potentiometric titrations as follows: (i) test substance in 0.01 *M* concentration in absolute ethanol titrated with an *N*/10 solution of KOH; (ii) aqueous solution of $CuCl_2$ in 0.005 *M* concentration against an *N*/10 solution of KOH; and (iii) a mixture of 0.01 *M* solution of the test substance and 0.005 *M*

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solution of $CuCl_2$ against an $N/10$ solution of KOH ($FeSO_4.7H_2O$ was tested against salicylanilide only). The results of the titrations with salicylanilide and 5-chloro-salicylanilide, in which pH vs. ml of KOH added was plotted, are shown in Fig. 1. Similar curves were obtained for the dichloro-salicylanilides. These curves were distinctively different from those obtained with the known chelating agent, glycine. In the case of the salicylanilides the curves for the mixture of reagents and the metal salts, unlike those in the case of glycine, appear to follow the individual curve for the reagent and the metal salt in turn. This is characteristic of the curves obtained with non-chelating compounds. The solubility effect of metallic ions on salicylanilide was determined by mixing a solution of the metallic ion with a solution of salicylanilide so that the final strength of the metallic ion was 0.0001 *M* and that of salicylanilide was 0.001 *M*. Usually two molecules of a chelating agent combine with one ion of metal, hence in these tests a fivefold excess of salicylanilide was present. These tests were repeated at a 10-fold higher dilution. For purposes of comparison, 8-hydroxyquinoline was tested under similar conditions. While 8-hydroxyquinoline gave colored precipitates with copper, and with ferrous and ferric iron, no such visible changes were observed with salicylanilide.

To determine if metallic ions could reverse the inhibitory effect of salicylanilide on *Trichophyton rubrum* and *Trichophyton mentagrophytes*, solutions of the metals in excess of the theoretical equivalent required to combine with the salicylanilide present were added to tubes containing the minimum inhibitory concentration ($M/20{,}000$) in 10 ml broth medium (2). Controls

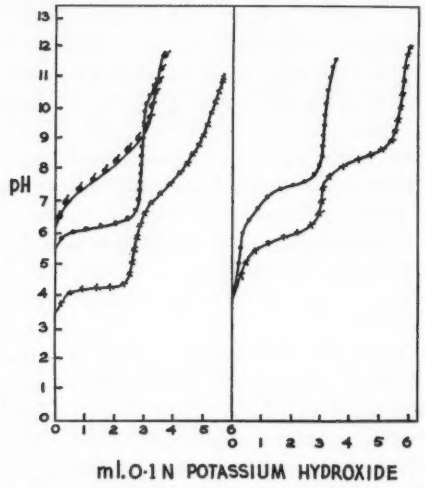

FIG. 1. Potentiometric titrations with salicylanilide and 5-chlorosalicylanilide to detect chelation.

♂ 0.01 *M* salicylanilide
• 0.005 *M* $CuCl_2$
× 0.01 *M* salicylanilide and 0.005 *M* $CuCl_2$
• 0.01 *M* 5-chlorosalicylanilide
× 0.01 *M* 5-chlorosalicylanilide and 0.005 *M* $CuCl_2$

containing the same concentrations of the metallic ions without salicylanilide, those containing only salicylanilide, and controls with neither salicylanilide nor the metal salt, were included in each test. After inoculation with 0.1 ml of a standardized conidial suspension of *T. rubrum* (2), the tubes were incubated for 3 weeks at 27° ± 2° C and observed for growth. Similar tests were also carried out using broth medium adjusted to pH 9 and 10 with *T. mentagrophytes* as the test organism (2). Since dermatophytic organisms are known to grow even at alkaline pH and since Pfeiffer and Glaser (6) reported the formation of a copper complex of salicylanilide under highly alkaline conditions an attempt was made therefore to obtain a reversal of the fungistatic action of salicylanilide in a medium of higher pH. The results obtained showed no reversal of the inhibitory effect of salicylanilide on *T. rubrum* in broth medium at pH 6.5–6.7 when the divalent metallic ions Cu, Co, Fe, Mg, Mn, Zn, Mo were used in concentrations of 0.005 *M*. A similar lack of reversal of the inhibitory effect of salicylanilide on *T. mentagrophytes* was obtained using the same divalent metallic ions at pH 9 and 10. It has also been observed that *T. mentagrophytes* is able to grow, although slowly, in a metal-deficient media (oxine treated) and that salicylanilide inhibited growth at the same concentration of *M*/20,000. This also suggests that the activity of salicylanilide may not be significantly related to the metal content of the medium. It cannot be considered from these results that the mechanism of action of salicylanilide involves chelation with free metallic ions in the growth medium as has been shown with 8-hydroxyquinoline (1, 7) and actidione (3).

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NOTES TO CONTRIBUTORS

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